I. The Office Action

The October 14, 2005 office action in this application (the "Office Action"):

1. noted applicant's election of Group I (claims 1-20, 32 and 33);

2. noted a reference cited in the April 10, 2002 IDS but not considered by the examiner;

3. objected to the lack of a description of drawings in the specification;

4. rejected claims 1-5, 7-17, 19-20 and 32-33 under 35 U.S.C. section 112, first paragraph;

5. rejected claims 1-2, 5-6, 8, 10-14 and 33 under 35 U.S.C. section 102(b) as being anticipated by the Schwartz (1998) reference;

6. rejected claims 1-2, 5-8, 10-14 and 33 under 35 U.S.C. section 103(a) as being unpatentable over Schwartz (1998) in view of U.S. patent 6,143,037;

7. rejected claims 1, 8 and 33 under 35 U.S.C. section 103(a) as being unpatentable over WO 94/24155, and;.

8. provisionally rejected claims 1-20 and 33-34 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-13 of copending application 10/929,040.

Applicants respond to the Office Action as follows.

II. <u>Prior Election with Traverse</u>

Numbered paragraph 1 on page 2 of the Office Action states that applicants have elected Group I (claims 1-20 and 32-33) without traverse.

Respectfully this is not correct as on page 3 of applicant's July 14, 2005 response to the June 20, 2005 office action in this application, applicants elected Group I (claims 1-20 and 32-33) with traverse.

III. Information Disclosure Statement

Numbered paragraph 3 on page 2 of the Office Action states that the IDS filed April 10, 2002 "continues not to be examined in its entirely". Please note that in no prior communication from the Office has an issue been raised or a comment made with regard to the IDS filed by applicants on April 10, 2002.

Numbered paragraph 3 on page 2 of the Office Action also states that for the IDS filed April 10, 2002 "There are several references that did not accompany the file". If the Office will identify these references applicants will provide them.

Numbered paragraph 3 on page 2 of the Office Action also states that Reference CD cited in the IDS filed April 10, 2002 has not been considered and invited applicants to resubmit this document. Enclosed is another copy of Reference CD. Please note that the last page of this publication provides an English abstract of the title and contents of reference CD.

IV. Objection to the Specification

The Office Action objected to the specification for lack of a description of drawings in the specification. Please note that this application does not have any drawings.

Related application serial number 10/929,040 (our docket 17326CIP3) was filed August 27, 2004 with 10 drawings. If the drawings from related application serial number 10/929,040 have become associated with this file the examiner is asked to kindly place such drawings with application serial number 10/929,040.

V. Section 112(1) Rejection

The Office Action rejected claims 1-5, 7-17, 19-20 and 32-33 under 35 U.S.C. section 112, first paragraph. The Office Action states that the specification is enabling for a method for treating a mammary gland disorder with Clostridial botulinum toxin A and Clostridial difficile toxin A.

The claims have been limited to a method for treating a mammary gland disorder with a botulinum neurotoxin toxin A, B, C, D, E, F or G. Support for such a claim limitation can be found from at least original claim 5 and page 31, lines 25-26 of the specification. Regarding enablement for such an amended claim it is well known that: "The botulinum toxins comprise a family of pharmacologically similar toxins that block acetylcholine release from peripheral nerves and cause a flaccid paralysis. All of the serotypes of toxin can poison humans and other animals..." (page 81, left hand side of Schantz, E.J., et al, *Properties and use of Botulinum toxin and Other Microbial Neurotoxins in Medicine*, Microbiol Rev. 56;80-99:1992) (copy enclosed).

Hence since all of the botulinum toxins act through the same physiological mechanism, and all of the botulinum toxins are disclosed in the original specification, use of all the botulinum toxins in the claimed method, not just botulinum toxin type A, is enabled by the specification. For these reasons the rejection should be withdrawn.

VI. Section 102(b) Rejection

The Office Action rejected claims 1-2, 5-6, 8, 10-14 and 33 under 35 U.S.C. section 102(b). Respectfully, the rejection is in error and should be withdrawn. The Schwartz (1998) reference discusses a muscle disorder not a mammary gland disorder. Thus, since the mammary gland is comprised of secretory glandular tissue a reference which discusses treatment of a muscle is not a relevant reference. Schwartz discuses treatment of a muscle which underlies but which is not a part of the mammary gland. Specifically, Schwartz discloses administration of a botulinum toxin into the latissimus dorsi muscle using EMG recording to insure that intramuscular injection was achieved. See page 188, right hand side column of Schwartz. The enclosed copy of Plate 160 from "Atlas of Human Anatomy" (Netter 1997) shows the location of the latissimus dorsi muscle. Clearly, the latissimus dorsi muscle is not a part of the mammary gland. Hence, Schwartz cannot anticipate claims limited to treatment of a mammary gland, as opposed to treatment of a muscle.

Additionally the claims have been limited to the scope of not rejected claim 3. For these reasons the rejection should be withdrawn.

VII. Section 103(a) Rejection

The Office Action rejected claims 1-2, 5-8, 10-14 and 33 under 35 U.S.C. section 103(b) as being unpatentable over Schwartz (1998) in view of U.S. patent 6,143,037. Respectfully, the rejection is in error and should be withdrawn.

The deficiencies of the Schwartz (1998) reference have already been discussed and Schantz appears to teach away from treatment of a mammary gland as opposed to intramuscular injection of a botulinum toxin.

U.S. patent 6,143,037 discloses a coated medical treatment device.

Since Schantz teach away from treatment of a mammary gland as opposed to intramuscular injection of a botulinum toxin, a combination of Schwartz and U.S. patent 6,143,037 cannot validly be made. Even if a combination of Schwartz and U.S. patent 6,143,037 could validly be made such a combination would merely teach intramuscular administration of a coated medical device, as opposed to the claimed invention. Additionally, these does not appear to be any motivation to combine Schwartz with U.S. 6,143,037 or any reason to believe that such a use of such a coated medical device would have a reasonable expectation of success with regard to the claimed invention.

Furthermore, claims 1, 11-12, 14 and 33 have been amended, claims 2 and 5 have been cancelled, and claims 6-7 and 10 have been amended to be dependant upon amended claim 1.

Further, the rejection appears to be based on "implantation of a botulinum toxin implant" (top of page 7 of the Office Action). Applicants have therefore cancelled claim 7.

For these reasons the rejection should be withdrawn.

VIII. Section 103(a) Rejection

The Office Action rejected claims 1, 8 and 33 under 35 U.S.C. section 103(b) as being unpatentable over WO 94/24155. This reference teaches use of Clostridium difficile toxin A, not the claimed use of a botulinum neurotoxin. It is well know that Clostridium difficile toxin A is (1) not a botulinum toxin and (2) not a neurotoxin. See eg the enclosed Bruggemann (2005) and Gerhard (2005) publications which state that Clostridium difficile is a enterotoxin or a cytotoxin which causes diarrhea and a form of colitis due to Rho enzyme inhibition, whereas botulinum toxin is a neurotoxin which causes botulism by inhibition of SNAP-25 mediated intracellular vesicle-cell wall docking.

Since WO 94/24155 does not teach or suggest use of a botulinum toxin the rejection should be withdrawn.

IX. Double Patenting

The Office provisionally rejected claims 1-20 and 33-34 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-13 of copending application 10/929,040. There is no claim 34 in this application.

An executed terminal disclaimer is enclosed so the provisional rejection should be withdrawn.
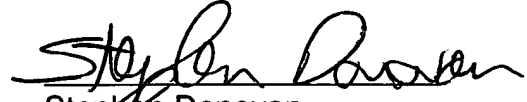
XI. <u>Cancelled Claims</u>

Applicants hereby cancel claims 2-3, 5, 7 and 16-17 without prejudice to further prosecution at a later date.

XI. Conclusion

All issues raised by the Office Action have been addressed. Examination and allowance of claims 1, 4, 6, 8-15, 18-20 and 32-33 is requested.

Respectfully Submitted,

Date: November 23, 2005

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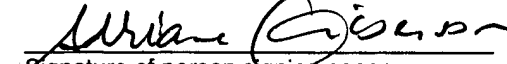2525 Dupont Drive
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<u>CERTIFICATE OF EXPRESS MAIL UNDER 37 C.F.R. § 1.10</u>

I hereby certify that this Transmittal Letter, the Response to Office Action and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date November 23, 2005 in an envelope as "Express Mail Post Office to Addressee" Mailing Label number EV 616125305 US addressed to Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Adriane Giberson
Name of person mailing paper

Date: November 27, 2005

Signature of person signing paper

PROGESTAGENIC ACTIVITY AND MECHANISM OF CONTRACEPTIVE ACTION OF MECYGEPRON

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and *I. S. Levina*

Progestagenic activity of a modified analog of progesteron-6α-methyl-cyclogexan-[1′, 2′, 16α, 17α]-pregn-4-en-3,20-dion including into mecygepron composition has been studied; its activity is proved to be 5 times as great as that of progesteron at any way of administration. Mecygepron affects processes of ovogenesis and early embryogenetic development in rats. A decreased number of washed out embryos is observed, while the number of lutein bodies specific for intact rats remains the same, decelerated rate of cleavage and increased number of unfertilized ova. In the mechanism of the changes observed an essential role plays a decreased secretion of lutropin resulting in a prolonged estrol cycle and, evidently, in intrafollicular overmaturation of the ova, which loose their ability to be fertilized. Besides, the disturbed hormonal balance under mecygepron influence can affect the character of the oviduct and uterine contents and, thus, prevent the normal development of the fertilized gametes.

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УДК 611.69-018.1-08 : 577.153.9]-08 : 599.323.4

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ЛОКАЛИЗАЦИЯ ХОЛИНАЦЕТИЛТРАНСФЕРАЗЫ В АЛЬВЕОЛЯРНОМ ОТДЕЛЕ МОЛОЧНОЙ ЖЕЛЕЗЫ БЕЛОЙ МЫШИ

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За последние десять лет доказан холинергический механизм регуляции секреторного процесса в альвеолах молочной железы [4, 8]. В связи с этим представляется важным выявление основных компонентов холинергической системы в их клетках. Поскольку присутствие холинэстеразы не служит однозначным показателем холинергических механизмов, оказалось необходимым продемонстрировать способность исследуемых структур к синтезу ацетилхолина. Поэтому мы провели гистохимическое исследование распределения холинацетилтрансферазы (фермента, катализирующего реакцию синтеза ацетилхолина) в альвеолярном отделе молочной железы белых мышей.

Материал и методика. Исследованы 5 белых мышей на 10—15-е сутки лактации. Животных декапитировали. Криостатные срезы (10 мкм) фиксировали в 1 % забуференном растворе формальдегида и подвергали гистохимической обработке для выявления холинацетилтрансферазы [11, 12, 15]. Для торможения эстераз срезы преинкубировали в растворе эзерина (10^{-3} М) и добавляли эзерин в инкубационную среду. Специфичность выявления холинацетилтрансферазы проверяли используя морин (2, 3, 4, 5, 7-пентагидроксифлавон) в концентрации 3×10^{-4} М [6, 9, 10] и 4-(1-нафтилвинил) пиридин в концентрации 5×10^5 М [13, 16], которые избирательно подавляют активность холинацетилтрансферазы не менее, чем на 60 %, причем морин использовали только в период преинкубации, так как добавление его в инкубационную среду приводило к образованию осадка.

Результаты исследования и их обсуждение. Как можно судить по рис. 1, клетки альвеолярного отдела молочной железы не содержат гранул продукта реакции, что свидетельствует о специ-

73

фичности примененных методик в отношении ацетил-КоА. В ходе реакции после отщепления ацетата от ацетил-КоА освобождаются SH-группы коэнзима А, которые и обеспечивают последующее образование продукта реакции. Многие внутриклеточные структуры содержат SH-группы, способные служить субстратом для развития реакции. Однако отсутствие реакции в выбранных нами условиях инкубации без ацетил-КоА показывает специфичность реакции в отношении сульфгидрильных групп коэнзима А. Наряду с ацетил-КоА другим обяза-
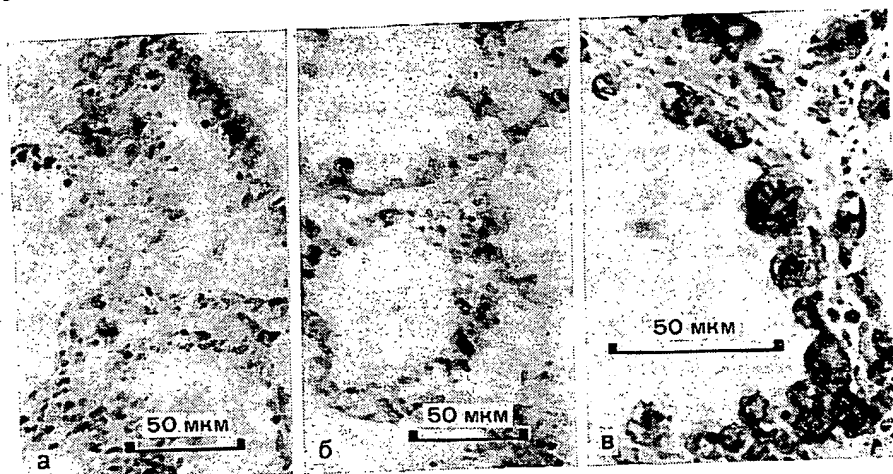

Рис. 1. Гистохимическое выявление холинацетилтрансферазы в тканях молочной железы белой мыши.

а — инкубация в среде, не содержащей ацетил-КоА, отсутствие продукта реакции в секреторных клетках альвеол; б — инкубация в среде в присутствии специфического ингибитора нафтилвинилпиридина, значительное подавление образования продукта реакции в секреторных клетках; в — инкубация в полной среде, различная локализация продукта реакции в секторных клетках альвеол.

Fig. 1. Histochemical revealing of cholinacetyltransferase in tissues of the white mouse mammary gland.

а — incubation in the medium not containing acetyl-CoA, absence of the reaction product in secretory cells of the alveoli; б — incubation in the medium, when a specific inhibitor naphtilvinilpyridine is present, an essential inhibition of the reaction product formation in the secretory cells; в — incubation in a complete medium, various localization of the reaction product in the secretory cells of the alveoli.

тельным субстратом реакции, катализируемой холинацетилтрансферазой, является холин. Инкубация в среде без холина дала результаты, которые в основном можно расценивать как положительную гистохимическую реакцию. Это выражается в присутствии типичных коричневых гранул в тех же структурах железистой ткани, которые давали реакцию при инкубации в среде полного состава. Следует, однако, отметить заметно меньшую интенсивность отложения осадка на срезах в среде, не содержащей холина. Подобное образование продукта реакции в бесхолиновой среде объясняется тем, что присутствие в ткани эндогенного холина обеспечивает возможность осуществления реакции [13, 14, 17]. В контрольных опытах исключение из инкубационной среды одновременно ацетил-КоА и холина дает отрицательные результаты.

Наиболее важным контролем для доказательства специфичности гистохимической реакции является демонстрация подавления активности исследуемого фермента при инкубации срезов в полной среде в присутствии специфических ингибиторов фермента. Нафтилвинилпиридин в концентрации 5×10^{-5}М тормозил образование конечного продукта реакции (см. рис. 1, б). Инкубация с другим специфическим ингибитором морином $(3\times10^{-4}$М) также подавляла реакцию. На основании проведенных контрольных экспериментов, можно считать, что распре-

74

деление осадка, образующегося в процессе гистохимической реакции, соответствует локализации в ткани активности холинацетилтрансферазы.

Обращает на себя внимание связь продукта реакции с секреторными клетками органа (см. рис. 1, в). Наиболее типична локализация фермента в их цитоплазме. Гранулы осадка обычно распределены срав-

Рис. 2. Локализация холинацетилтрансферазы в структурах альвеолярного отдела молочной железы белой мыши.
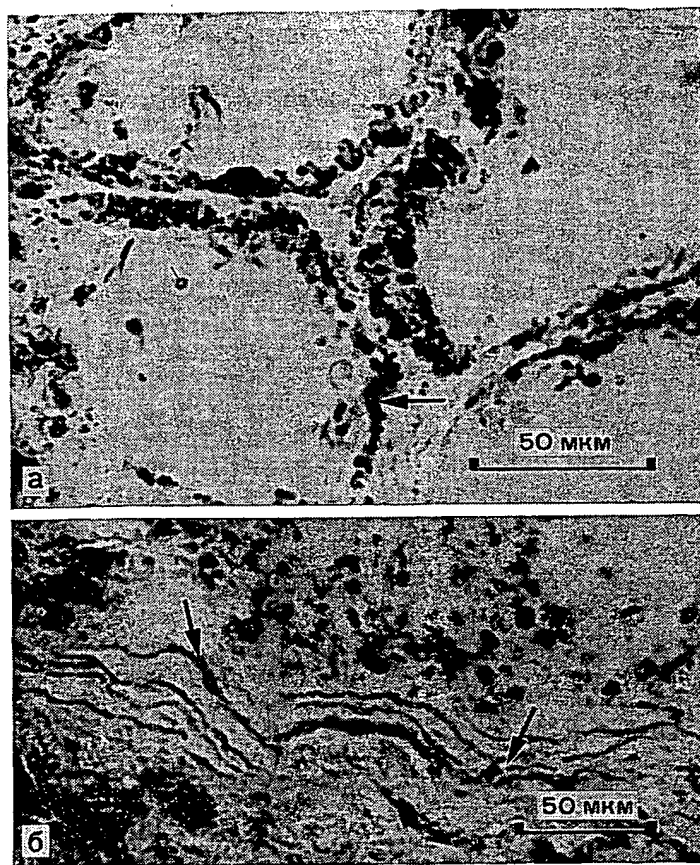
а — активность фермента в зоне секреторных клеток и в базальных участках альвеол, соответствующих расположению миоэпителиальных клеток (стрелка); б — активность фермента в отдельных нервных волокнах подвздошно-подчревного нерва молочной железы (стрелки).

Fig. 2. Cholinacetyltransferase localization in structures of the alveolar part of the white mouse mammary gland.

a — activity of the enzyme in the secretory cells zone and in the basal areas of the alveoli corresponding to arrangement of the myoepithelial cells (arrow); б — activity of the enzyme in separate neural fibers in the ileo-hypogastric nerve of the mammary gland (arrows).

нительно равномерно, однако в некоторых клетках плотность продукта реакции больше в апикальной или базальной области (рис. 2, а). Встречаются также клетки, в которых активность фермента низка (за границами чувствительности метода). Реакция в ядерных структурах обнаружена не во всех клетках (см. рис. 1, в). Отдельные участки базальной области некоторых альвеол тоже содержат продукт реакции, что позволяет предполагать возможное выявление активности фермента в миоэпителиальных клетках.

Привлекает внимание положительная реакция в нервных волокнах подвздошно-подчревного нерва молочной железы (см. рис. 2, б). Выявление активности фермента именно в этом нерве согласуется с фактом об освобождении ацетилхолина в перфузат при электрической стимуляции нерва [3]. Тесное соседство подвздошно-подчревного нерва со структурами альвеолярного отдела молочной железы предполагает существование нервных влияний как на клетки альвеол, так и на клеточные элементы межальвеолярной соединительной ткани. Это предположение подтверждается опытами, в которых рассматриваются изменения мембранного потенциала секреторных клеток при электрическом раздражении подвздошно-подчревного нерва молочной железы, идентичные изменениям мембранного потенциала, развивающимся в ответ на апликацию раствора ацетилхолина (1×10^{-6} г/мл) [7]. Кроме того, электрическая стимуляция этого нерва вызывает отчетливую вазодилатационную реакцию. Дальнейшие исследования, по-видимому, смогут подтвердить существование холинергических влияний на другие процессы, характерные для деятельности клеток альвеолярного отдела.

Расположение активности фермента дает основания считать основным местом образования ацетилхолина цитоплазму секреторной клетки. В то же время изучение субклеточной локализации холиновых эфиров в изолированных секреторных клетках молочной железы коров показало, что некоторую их часть содержат клеточные ядра [4], что подтвердили и результаты данного исследования. Полученные данные о наличии ферментативной системы синтеза ацетилхолина в альвеолярном эпителии молочной железы подтверждаются доказательствами возможности синтеза ацетилхолина в не нервных тканях и клетках [1, 4, 18]. Ацетилхолин, образующийся внутриклеточно, может выступать в роли местного гормона, осуществляющего регуляторные воздействия на различные функции клеток [1, 19].

На переживающей тканевой культуре молочной железы мышей после стимуляции экструзии секрета окситоцином были выявлены ритмические изменения содержания тканевого ацетилхолина, развивающиеся синхронно с другими показателями функциональной активности секреторных клеток [7]. При этом в практически идентичных условиях (даже в пределах одного среза) в молочной железе в различных альвеолах отмечается существенная гетерогенность распределения холинацетилтрансферазной активности, что, по нашему мнению, служит показателем функциональных различий, обусловленных асинхронностью прохождения альвеолами стадий секреторного цикла. Это позволяет объяснить ряд холинергических реакций органа, развивающихся в результате сокращений миоэпителиальных клеток как при действии окситоцина, так и при гиперкалиевой и электрической деполяризации. Блокада реакций секреторных клеток холинолитиками — атропином и сукцинилбисхолином [8], а также развитие вазодилатации при сокращениях миоэпителиальных клеток [2] позволяет обосновать возможность их взаимодействия с секреторными клетками, опосредованного холинергическим механизмом. Это подтверждается и результатами данной работы.

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ф и р о в Л. Н. и Ра
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LOCALIZATION OF CHOLINACETYLTRANSFERASE IN ALVEOLAR PART OF THE WHITE MOUSE MAMMARY GLAND

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The light optic investigation has been performed on the 10th — 15th days of lactation. The enzymatic activity has been determined by Burt method (1971). The product of histochemical reaction is revealed in the secretory cell and in the basal parts of the alveoli, that corresponds to location of myoepithelial cells. Localization of the reaction product in the secretory cells of the neighbouring alveoli is heterogenous. The enzyme of acetilcholine synthesis is revealed along the course of single neural fibers included into the composition of the ileo-hypogastric nerve of the mammary gland. The results obtained, together with the data of certain physiological investigations make it possible to substantiate participation of the cholinergic mechanism in the alveolar cells activity and consider participation of the cholinergic process in intercellular interactions, that ensure functional conjugation of myoepithelial and secretory cells.

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Properties and Use of Botulinum Toxin and Other Microbial Neurotoxins in Medicine

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INTRODUCTION

The eminent physiologist Claude Bernard wrote in his classic work entitled *Experimental Science* (8), "Poisons can be employed as a means for the destruction of life or as agents for the treatment of the sick." He went on to explain how certain toxins and poisons were valuable tools for analyses of the most delicate phenomena of living structures. Although several toxic substances of plant and animal origin were used in medical practice during his time, in recent years a great multitude of poisonous substances from plants, animals, and microorganisms are now finding use in studies on animal physiology and some are used medicinally in humans.

In December 1989 the U.S. Food and Drug Administration licensed botulinum toxin type A as an orphan drug for the treatment of the human muscle disorders strabismus, hemifacial spasm, and blepharospasm in patients 12 years of age and older, by direct injection of the toxin into the hyperactive muscle. Botulinum toxin is also being used experimen-

tally for the treatment of a number of other dystonias and movement disorders (25, 98, 191). The use of the toxin for human treatment came about over 20 years ago through the collaborative work of Alan B. Scott and E. J. Schantz. The treatment of neurological disorders with botulinum toxin type A has opened a new field of investigation on the application of the toxin to nerve and muscle tissue in the human body.

Various microbial neurotoxins are being used to understand the physiology of the nervous system and may have potential value in the treatment of certain types of muscular disorders through modification of nervous stimulation of muscle activity. Well-characterized microbial neurotoxins for this purpose include the neurotoxic proteins from *Clostridium botulinum* and *Clostridium tetani* and the low-molecular-weight neurotoxins saxitoxin and tetrodotoxin, from certain species of dinoflagellates and bacteria (Table 1). These toxins affect muscular activity by their direct action on the nervous system; for example, botulinum and tetanus toxins affect activity by a presynaptic block of the release of neurotransmitters, and saxitoxin and tetrodotoxin do so by altering the action potential at the voltage-gated sodium channels of neurons. These toxins differ from many other

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TABLE 1. Approximate relative toxicities of microbial neurotoxins

Toxin	Minimum lethal dose in mice (µg/kg)	Mol wt
Botulinum toxin, type A, crystalline	0.00003	900,000
Tetanus toxin, crystalline	0.0001	150,000
Saxitoxin (free base)	9	299
Tetrodotoxins	8–20	319

microbial toxins such as diphtheria and cholera enterotoxins in that they exhibit relatively little cytolytic or cytotoxic activity. This review describes properties of presently known neurotoxins that are obtained from microorganisms and that, through their physiological action, may be valuable in medicine and pharmacology, particularly botulinum toxin type A, the only toxin which is presently being used for the treatment and relief of several human dystonias. It also points out the need for research on methods for culturing, purification, genetic expression, and preservation of these toxins applicable to their use for human treatment.

MICROBIAL NEUROTOXINS THAT BLOCK NEUROTRANSMITTER RELEASE

Properties of Botulinum Toxin Type A Relevant to Its Use in Medicine

Developments leading to the use of the toxin for human treatment. Botulinum neurotoxins are produced by certain strains of the bacterial species. *C. botulinum*, *Clostridium butyricum*, *Clostridium baratii*, and *Clostridium argentinense* (86). The toxins are classified into seven serotypes, A through G, on the basis of their immunological properties. The botulinum neurotoxins comprise a family of pharmacologically similar toxins that block acetylcholine release from peripheral nerves and cause a flaccid paralysis. All of the serotypes of toxin can poison humans and other animals, but type A has caused the severest illness and many deaths from food-borne botulism and is the best-characterized botulinum toxin. Crystalline type A toxin is the serotype that is currently being used in therapeutic applications. The following sections describe the basic properties of botulinum toxin type A and the development of the toxin as a drug.

Investigations into the use of botulinum toxin type A for the treatment of hyperactive muscle disorders originated over 20 years ago through a fortunate set of circumstances and the ingenuity of Alan B. Scott, a surgeon at the Smith-Kettlewell Eye Research Institute in San Francisco. He contacted one of us (E.J.S.) regarding the availability of a toxic substance that might be injected into a hyperactive muscle and thus serve as an alternative to surgery for the treatment of strabismus, a condition in which the eyes are out of alignment. In my research on microbial toxins I had on hand highly purified crystalline type A botulinum toxin, produced by *C. botulinum*, and saxitoxin, the potent poison produced by the dinoflagellate *Gonyaulax catenella*. The mechanisms of action of these toxins had been known for many years (29, 33, 63, 103), and their possible use in the treatment of a hyperactive muscle was apparent but had never been tested. No record of such use in animals or humans was available. Both botulinum toxin and saxitoxin cause flaccid paralysis of skeletal muscle as a result of action

on the nervous system. Botulinum toxin type A appeared to be the toxin of choice for human treatment on the basis of animal studies and accidental cases of human food poisoning in which the paralytic action on survivors lasted for many weeks whereas recovery from saxitoxin poisoning took only a few days for survivors. We therefore began our collaboration on this work by using botulinum toxin experimentally on rhesus monkeys, in which Dr. Scott surgically produced a condition similar to strabismus. With the properly determined dose of botulinum toxin injected into the more active muscle, proper alignment of the eyes was achieved.

After 10 or more years of successful experiments on monkeys, the FDA granted Dr. Scott permission to treat strabismus in human volunteers. Strabismus in humans is a disorder of vision due to turning of one or both eyes from the normal position for binocular vision and is caused by hyperactivity of one or more muscles controlling eye position. This condition in humans usually is corrected by surgery, which involves cutting away a sufficient portion of the hyperactive muscle to allow the eye to assume its normal position. Successful human treatment with the toxin involved injecting measured amounts of the toxin, under carefully controlled conditions using electromyography, directly into the hyperactive muscle pulling the eye out of alignment. Injection of botulinum toxin weakened the overactive muscle, enabling compensation by the weaker one and resulting in permanent eye alignment after a period of temporary paralysis (192). The clinical work was first reported by Scott in the 1980s (188, 189), and the properties of the toxin in relation to its use in medical treatment was reported by Schantz and Scott in 1981 (180).

Special considerations on the preparation and maintenance of botulinum toxin type A for human treatment. Although the original toxin on hand and that prepared for the monkeys was sufficient, the toxin to be used for the human trials had to be prepared under more specific conditions that would, from best judgment, meet approval by the FDA. Botulinum toxin is the first microbial protein to be used via injection for the treatment of human disease. There was no precedent for the use of a microbial toxin in this manner, and protocols for this work had to be implemented. The important considerations regarding the toxin were its purity and dose on injection. The production by culturing and the purification had to be carried out so that the toxin was not exposed to any substance that might contaminate the final product in trace amounts and cause undue reactions in the patient. These restrictions required culturing in simplified medium without the use of animal meat products and purification by procedures not involving synthetic solvents or resins. Another concern was the problem of long-term stability of the toxin so that a supply was always available. Dilution of the toxin from milligram quantities to a solution containing nanograms per milliliter presented a problem because of the rapid loss of specific toxicity on such great dilution. Toxin can be diluted in pyrogen-free water or saline if used immediately for treatment, but stabilization of the toxin for longer periods requires the presence of another protein such as gelatin or albumin (173, 177). Although the commercial botulinum type A product is prepared in the presence of human serum albumin, the use of human serum albumin presents potential problems in that certain stable viral agents carried through from donors could contaminate the toxin. These and other concerns about the preparation and use of the toxin for human treatment are reviewed and discussed in the following sections.

Mechanisms of action of botulinum toxin. The primary

structure of the neurotoxin is such that the resulting shape (secondary and tertiary structures) causes a highly specific binding and block of acetylcholine release at myoneural junctions. Botulinum toxin is toxic to all vertebrates through weakening of skeletal muscle, and death may come about through paralysis of the muscles of respiration. Van Ermengen (226) considered that the toxin acted on the central nervous system, but it was later shown that the action is peripheral rather than central (50, 60). Most early studies on the mechanism of action of botulinum toxin were carried out with type A crystalline toxin (29, 33, 79). Botulinum toxin blocks cholinergic transmission at all cholinergic synapses in the peripheral nervous system, but conduction along axons is not affected (79). The chemical denervation lasts for several months, and recovery of neurotransmission and muscle activity requires sprouting of new nerve endings and functional connections at motor end plates. The biochemical mechanisms of botulinum toxin in skeletal neuromuscular preparations, brain synaptosomes, chromaffin cell cultures, spinal cord cell cultures, and *Torpedo* and *Aplysia* preparations have been reviewed within the last 10 years (45, 81, 137, 158, 168, 200).

Preparation and properties of botulinum toxin type A for clinical use. The Food Research Institute, University of Wisconsin, has been involved in the production of crystalline toxin pertaining to food safety for many years, and small amounts of this toxin were used for the work on monkeys. However, the toxin that was to be used for human treatment by injection required special considerations, and preparation and purity of the toxin became essential (180). The type A toxin Hall strain was chosen for production of toxin because it consistently produced high levels of toxin (1 to 4 million mouse 50% lethal doses (MLD_{50}) per ml of culture broth). It was originally obtained from J. H. Mueller of Harvard University (118), and was developed at Fort Detrick, Md., by screening for high toxin production. Toxin is produced in a nutritive medium consisting of a casein digest, yeast extract, and dextrose at pH 7.3. Following inoculation, growth is usually complete in 24 to 36 h, at which time cells undergo lysis. Complete lysis and clearing of the culture take 2 to 3 days. The toxin is liberated during lysis and is activated by proteases present in the culture broth that convert a poorly active protoxin to the highly potent toxin.

The first successful attempt at purification of type A toxin from culture broths was accomplished by Snipe and Sommer (203) at the Hooper Foundation at the University of California in 1928, when they showed that 90% of the crude toxin could be precipitated from the spent culture fluid by the addition of acid to pH 3.5. About 20 years later, Lamanna et al. (113), starting with the precipitate, obtained the toxin in crystalline form, and then Duff et al. (59) improved the method; the improved method is the basis for the present procedure for purification. The purification of botulinum toxin type A in our laboratory for human use was designed to be carried out by the simplest procedures and avoided exposure to substances such as added enzymes or columns of synthetic resins, used in some methods, that could contaminate the preparation and be carried into the final injected preparations. It is briefly described as follows. The type A toxin in the spent broth was first precipitated by adjustment to pH 3.5 with acid; 90% of the toxin was recovered in the precipitate. The precipitate was washed with water, and the toxin was extracted with 1 M salt solution at pH 6.5 and reprecipitated with acid at pH 3.7. The toxin was extracted from this precipitate with 0.05 M sodium phosphate buffer at pH 6.8, precipitated in 15% ethanol at −5°C, redissolved in

phosphate buffer, and crystallized in 0.9 M ammonium sulfate. This simplified procedure yields, for example, 60 to 70 mg of small, white, needle-shaped crystals (0.1 to 0.2 mm in length) from a 12-liter culture (15 to 17% recovery). Recrystallization under the same conditions yields 20 to 25 mg of crystalline toxin.

The crystalline type A toxin contains 16.2% nitrogen and, as far as is known, is composed only of biologically active amino acids (32, 207) for both the neurotoxin and the nontoxic proteins. The isoelectric point of the crystalline type A toxin is pH 5.6. Under slightly acidic conditions, pH 3.5 to 6.8, the neurotoxic component of 150,000 M_r is bound noncovalently to the nontoxic proteins in such a manner as to preserve or help stabilize the second and tertiary structures upon which toxicity is dependent. Under slightly alkaline conditions (>pH 7.1) and in the blood and tissues of animals and humans, the neurotoxin is released from the toxin complex. RNA is also associated with the toxin complex but has no known role in activity or stability.

The molecular weight of crystalline toxin was initially shown to be 900,000 M_r on analysis in the ultracentrifuge at pH 3.8 to 4.4 (161, 173, 181, 229). Putnam et al. (161) showed that on electrophoresis, the crystalline toxin moved as a single substance with a molecular weight of 900,000. Lamanna et al. (115) discovered that purified type A toxin could be separated into nontoxic and toxic components, when they found that a nontoxic component precipitated erythrocytes leaving the toxin in solution. Wagman and Bateman (229) also showed that the toxin moved in the ultracentrifuge as a single substance with a sedimentation coefficient of 19S at pH 5.6, but at pH 7.3 the toxin component (neurotoxin) dissociated and moved as a much smaller molecule (7S). Later DasGupta and Boroff (46) showed that at alkaline pH the neurotoxin could be separated from the nontoxic proteins by column chromatography.

On diffusion in agar gel at pH 4.2 the crystalline toxin moves as a single substance with a coefficient (D) of ca. 2 × 10^{-7} cm^2 s^{-1} (161, 178). However, at pH 7.3, near to the pH at which the neurotoxin and nontoxic components dissociate, the diffusion rate of the neurotoxin increased to ca. 8 × 10^{-7} cm^2 s^{-1}, much higher than the rate expected for a globular protein molecule of 150,000 M_r (178). Diffusion depends to a great extent on the shape of the molecule, and the toxin may take on a threadlike structure that would diffuse faster than a globular structure.

The biological activity (toxicity) of the toxin, like many other biologically active proteins, is due to the spatial or conformational structure of the neurotoxin molecule (173, 182). The nontoxic proteins bound to the neurotoxin apparently play an important role in maintaining the toxic shape of the neurotoxin. Careful handling of purified toxin is therefore important for maintenance of stability. Botulinum toxin type A is readily denatured by heat at temperatures above 40°C, particularly at alkaline pH. Solutions of the toxin lose toxicity when bubbles form at the air/liquid interface causing stretching and pulling of the neurotoxin out of its toxic shape (173). This denaturation also takes place in an atmosphere of nitrogen or carbon dioxide. Dilution to extremely low concentrations (nanograms per milliliter) also tends to decrease the stability of the neurotoxin, but this can be prevented by diluting with a buffered solution (at pH 6.8 or below) containing another protein such as gelatin and certain albumins such as bovine or human serum albumin. When the pH is raised above 7.3, the neurotoxin is liberated, which is very labile. Because of its lability the neurotoxin is not practical for medical applications.

Crystalline botulinum toxin type A was the first microbial protein of this complexity to be considered an injectable substance by the FDA, and it was necessary to set down specifications for toxin quality. The following properties of the crystalline toxin obtained from many batches were found to be of the highest-quality toxin and were used for evaluation of batch 79-11, which was used in initial studies in humans and later licensed by the FDA: (i) a maximum absorbance at 278 nm when dissolved in 0.05 M· sodium phosphate buffer at pH 6.8, (ii) an A_{260}/A_{278} ratio of 0.6 or less, (iii) a specific toxicity for mice of $3 \times 10^7 \pm 20\%$ MLD_{50} per mg, and (iv) an extinction coefficient (absorbancy) of 1.65 for 1 mg of toxin per ml in a 1-cm light path.

The purity of the crystalline toxin cannot be defined strictly in terms of percent purity because of small amounts of undefined material absorbing at 260 nm, most probably nucleic acid material, which associates with the toxin during culturing and is carried through the purification and crystallization procedures. This property is peculiar to certain crystalline proteins, in contrast to the crystallization of other simpler organic substances. We have based the quality of the toxin on obtaining as low an A_{260}/A_{278} ratio as possible, near to 0.55. Toxin from the first crystallization has a ratio close to 0.6, and on the second crystallization it should be reduced to about 0.55, which is considered representative of high-quality toxin. A third crystallization may reduce the ratio slightly but at a cost of yield, because only one-third to one-half of the toxin is recovered on each crystallization. Other crystalline proteins such as human and bovine serum albumins have absorbance ratios close to 0.5 (232). If it is assumed that the absorbing material at 260 nm is nucleic acid with an extinction of 20 per mg (12 times that of the toxin), the proportion of nucleic acid in a preparation with a ratio of 0.6 would be less than 0.1%.

Another test of purity and consistency for each batch of type A toxin is the banding pattern on solution electrophoresis and gel electrophoresis with crystalline toxin and reduced crystalline toxin. At or below the isoelectric point of 5.6, the toxin moves as a single homogeneous substance, of 900,000 M_r. Toxin reduced with sulfhydryl reagents shows the distribution after electrophoresis of the nontoxic components along with the neurotoxin subunits of 100,000 and 50,000 M_r. Electrophoresis carried out on several batches of crystalline toxin showed that the toxin is judged very similar for each batch (101).

The specific toxicity of a high-quality preparation of crystalline toxin should be 3×10^7 MLD_{50} (±20%) per mg. The number of milligrams for this determination is based on the A_{278}, using the extinction of 1.65 to convert to milligrams of toxin ($A_{278}/1.65$ = milligrams of toxin [±3%]) (207). Because the immunological properties of type A toxin are independent of its toxic properties, the only means of evaluating the potency or acetylcholine-blocking power of the toxin is an animal assay (176). The mouse assay for toxicity determination may vary depending on the species of mice, their condition, and the conditions under which the assay is carried out. To minimize the variability, it is recommended that the mouse assay be carried out on any preparation used for human treatment with the use of a reference standard of type A toxin as described by Schantz and Kautter (177). There is no known chemical, physical, biological, or immunological test available that can replace the mouse test for toxicity evaluation.

An important factor in the medical use of botulinum toxin is a method of storage for retention of toxicity. The crystalline toxin formerly provided for reference in food assays was dissolved and stored in 0.05 M sodium acetate buffer (pH 4.2) at 4°C (177), in which it retained toxicity for 1 to 2 years before a significant loss (20%) could be detected by mouse assay. The difficulty with storing the toxin in acetate buffer is that freezing causes complete detoxification and reliance on storage at 4°C without danger of freezing is not practical under certain circumstances. However, we have found that the most satisfactory method of storage is to leave the crystalline toxin at 4°C in the mother liquor of the second crystallization, in which toxicity was retained for 10 or more years. Retaining stability is important because it makes available a bulk supply of toxin to draw from over an extended period. When the FDA approved experimental trials on human volunteers, a large batch of crystalline toxin was prepared in November 1979 (designated 79-11) expressly for the human trials; 100 mg was supplied to Alan Scott and 50 mg was retained in storage at the Food Research Institute. This batch has been the sole source of botulinum toxin type A accepted by the FDA for human treatment and has been used by many physicians throughout the United States and some foreign countries. However, some loss in toxicity has occurred in batch 79-11, and we recommend that fresher batches of toxin periodically be prepared to avoid detrimental changes that may occur on aging. Crystalline type A toxin prepared in our laboratory does not appear to differ in potency or clinical efficacy from type A toxin prepared in England by using anion-exchange chromatography and RNase treatment (133, 222). However, we do not recommend the use of methods of purification involving enzymes, various exchangers, or synthetic solvents because of the chance of contamination.

Preparation of the toxin for dispensing as a drug and compatible for injection into muscle required (i) dilution in a suitable medium for stability of toxicity, (ii) filtration for sterility, and (iii) drying. Diluting a solution of botulinum toxin type A from a concentration of 1 or 2 mg/ml to nanogram concentrations causes detoxification unless another protein is added for protection. Gelatin at 2 to 3 mg/ml is generally used at pH 6.2 in the standard procedure for the mouse assay for toxin in foods (177). Bovine serum albumin has been used at 2 to 3 mg/ml in acetate buffer at pH 4.2 for good stability (177), and human serum albumin was adopted for medical use. Filtration in the presence of additional protein can be carried out successfully to remove bacterial contamination without loss of toxicity. However, drying, which would have many advantages in long-term stability, under the conditions with human serum albumin at pH 7.3 resulted in a substantial loss (50 to 90%) of toxicity. This loss of toxicity is a very important consideration because of the possibility that the inactivated toxin will form a toxoid and immunize the patient against the toxin on continued use. Various methods of drying, particularly lyophilization, resulted in such losses. Experience with the toxin has proved that stability of toxicity is dependent on low pH (<7), but such low pHs are not compatible with injections into muscle tissue. A significant problem is the development of a medium and conditions to overcome the losses on drying, and research for this purpose is being carried out in our laboratory.

Therapeutic applications of botulinum toxin. Clinical studies have indicated that toxin injections can provide profound symptomatic relief for humans suffering from a wide variety of disorders characterized by involuntary movements of muscle groups (Table 2), particularly those involved in focal or segmental dystonias (25, 64, 92, 127). In 1911, Oppenheim (151) introduced the term "dystonia musculorum defor-

TABLE 2. Focal dystonias and involuntary movement disorders
successfully treated with botulinum toxin type A[a]

Condition	Symptoms of disease
Strabismus	Crossed eyes
Blepharospasm	Spasmodic eye closure
Hemifacial spasm	Facial twitching and spasms
Eyelid disorders	Inward turning of eyelid
Spasmodic torticollis	Abnormal movements or twisting of the neck and head
Oromandibular and lingual dystonia	Sustained mouth closure or lingual muscle contractions
Focal dystonias of the hand	Writer's cramp, musician's cramp, hand and arm muscle spasms
Spasmodic dysphonia	Uncontrolled vocal fold spasms
Other voice-disorders	Vocal tremor, stuttering
Neurogenic bladder	Abnormal urinary control; results from spinal cord injury
Anismus	Uncontrollable anal sphincter contraction
Limb spasticity	Occurs following stroke and other neurological disorders including cerebral palsy

[a] Listed approximately in decreasing order of numbers of patients treated (25, 98, 179, 191).

mans" to describe children who had movement disorders such as twisted postures, bizarre walking with bending and twisting of the torso, and severe muscle spasms. Oppenheim pointed out that progression of symptoms often resulted in fixed postural deformities. Dystonia is currently defined as "a syndrome of sustained muscle contractions, frequently causing twisting and repetitive movements or abnormal postures" (64, 127). Dystonia can affect all regions of the body (127). Many patients with dystonias have been diagnosed as experiencing psychological stress and referred for psychological therapy (64), but were later found to suffer from specific neurological diseases (64, 127). Adult onset of focal or segmental dystonias (which affect only one or a few muscle groups) are more common than generalized dystonias (64, 127). A study in Minnesota estimated the prevalence of various dystonias to be 391 per million population (147). Focal dystonias may spread and lead to generalized dystonias, in which several muscle groups are involved. Focal dystonias progressed to generalized conditions in nearly 60% of affected children (onset before age 13) and in about 3% of adults (onset after age 20) (127).

Crystalline botulinum toxin has had great benefit in the treatment of involuntary muscle conditions, and injection of toxin is now considered the most effective treatment for a variety of focal dystonias (25, 98, 191). On injection the toxin acts directly or indirectly to alleviate conditions that result from muscle hyperactivity. Direct paralysis of target muscles is desired for certain indications including blepharospasm, torticollis, and other focal dystonias. Depending on the syndrome, toxin injection generally relieves undesired muscle movement for a few months, after which the abnormal movement returns and repeated injections are required. Paralysis of certain muscle groups can also lead to secondary desired effects (191). For instance, paralysis of a hyperactive muscle enables compensation by a weaker muscle, as in treatment of strabismus and certain limb muscle spasmodic disorders. In these conditions, the balancing of agonist and antagonistic muscle systems is the desired effect (191).

Strabismus was the first syndrome for which botulinum toxin therapy was introduced as an alternative to surgery

(188, 189, 192). Botulinum toxin is usually injected into the recti muscles with a Teflon-coated needle and electromyographic guidance to ensure accurate placement in the muscle; this is usually an office procedure. The toxin evokes a temporary denervation and muscle weakening, allowing the globe to return to normal alignment. Although botulinum toxin will not replace conventional surgical treatment, it has proved to be a useful adjunct to surgery in certain cases (189, 191).

Botulinum toxin is being used primarily for the correction of focal dystonias and other regional movement disorders. One syndrome approved for treatment is essential blepharospasm, in which persons suffer from involuntary eyelid closure. Blepharospasm is often accompanied by involuntary movements of head and neck muscles, a condition known as Meige syndrome (98). Meige syndrome manifests as uncontrolled blinking (blepherospasm) plus involuntary facial grimacing, frowning, facial contortions, spasmodic speech, and neck pulling (spasmodic torticollis) (24, 25). The age of onset of blepharospasm is often 50 to 70 years, and the syndrome may progress to other muscle regions. Injections of botulinum toxin type A into the orbicularis oculi muscle has given clinically significant benefit in 70 to 90% of more than 8,000 treatments (98). In most patients, the latency period from injection to onset of improvement was 2 to 5 days and relief persisted for an average of 3.5 months. The average dose was ca. 20 U (191). In some treatments, toxin diffused to neighboring muscles and caused temporary ptosis. Some patients have received repeated injections for 7 years or more, and no adverse long-term effects have been observed.

Hemifacial spasm is an often disfiguring syndrome characterized by involuntary movement of facial muscles controlled by the seventh facial nerve. Patients often find the movements disfiguring and socially and functionally incapacitating (25). Treatments with neuroleptic medications have been entirely ineffective. Injection of botulinum toxin (generally 10 to 20 U) has relieved hemifacial spasm in more than 90% of the patients treated. Most patients experience relief for 3 to 4 months, after which repeated injections have provided long-term relief in most individuals.

Spasmodic torticollis (cervical dystonia) is a dystonia affecting neck muscles and causing the head to involuntarily deviate in any direction (25, 75, 98, 208). It is among the most common dystonias, and the spasmodic contractions can cause posture deformity, head tremors, and pain. Over 1,000 cases of spasmodic torticollis have been treated, and the studies have reported improvement in 50 to 90% of the patients, depending on the dose and placement of the toxin. Comparatively large doses of botulinum toxin are used for injection at multiple sites. The larger quantities of toxin can diffuse to neighboring muscles, causing ptosis and other side effects.

Certain other diseases involving involuntary muscle movements have been successfully treated with botulinum toxin in a limited number of patients (reviewed in references 25, 98, 191, and 218). These include writer's and musician's cramps, hand tremors, spasmodic dysphonia and other laryngeal dystonias, neurogenic bladder as a result of spinal cord injury, spasms of the rectal sphincter (anismus), limb muscle spasms following stroke, leg spasms from multiple sclerosis, and spasticity in children with cerebral palsy. Botulinum toxin could potentially benefit humans who suffer from a variety of other hyperkinetic movement and muscle tone disorders including tics, tremors, bruxism, and pain brought on by muscle spasms (25, 98, 99, 218).

Although botulinum toxin is currently used for treatment of regional muscle groups, limited success has also been achieved with patients who suffer from hyperactivity of several muscle groups. Botulinum toxin has found limited use in tardive dyskinetic syndrome (221), a chorea marked by irregular dystonic movements and postures that can develop in mentally ill patients after treatment with neuroleptic medications. Some of these patients experience marked distress and suffer from disparate spasmodic disorders including repetitive blinking, backward arching of the head and trunk (retrocollis), rocking of the body, mouth grinding (bruxism), and involuntary voice sounds and grunting. In a pilot study, four patients were injected in diverse muscles and marked improvement was found in 2 weeks in all four individuals. Not all movement disorders in these patients improved, but several did including retrocollis, mouth control, and bruxism. Treatment of tardive dyskinetic syndrome by chemical denervation with botulinum toxin is complex because it involves different muscle groups. The strategy has been to focus toxin injection on the most involved muscle groups.

Generalized dystonias such as those observed in Parkinsonism present difficult problems for treatment because of the many muscles involved, but it is possible that if a proper method of administration could be worked out, these generalized conditions could be treated with toxin. One possible but untried route is the administration of low intravenous doses by which the toxin would spread regionally to many muscles.

Side effects of botulinum toxin. No adverse clinical effects of botulinum toxin have been found in patients who received low doses of botulinum toxin, e.g., ≤20 U. Single-fiber electromyography analysis has shown that injection of relatively large quantities of botulinum toxin (140 to 165 U) leads to toxin spread, weakening of distant muscles, and uncharacterized subclinical effects (116).

The primary side effect associated with local injections of botulinum toxin is weakening and ptosis of nearby muscles. One of the most prevalent and disturbing side effects is dysphagia, or the inability to swallow, and several patients have experienced upper airway obstruction after treatment with relatively high doses (>150 U) of botulinum toxin (25, 208). Dysphagia may be related to generalized weakness and inability to hold the head erect (75) or to weakening of muscles involved in swallowing. It may also be related to the dose and injection strategy used. To prevent dysphagia, Borodic et al. (23, 24) have recommended, on the basis of studies of toxin diffusion in tissues, the use of ≤100 U per treatment injected into several sites. Further research is needed to identify the lowest dose of toxin and sites of injections that will produce the desired control and prevent migration of toxin to neighboring muscle groups. Local side effects could be increased in patients who are being treated with drugs other than botulinum toxin that affect neuromuscular transmission (4).

There is interest among physicians in developing methods to prevent the spread of toxin to neighboring muscles. Scott (190) demonstrated that injection of antitoxin at the correct time following toxin injection partially prevented toxin migration. The currently available equine antitoxin could lead to undesirable reactions in some patients, and it would be valuable to have a source of human antibodies. In January 1991, human immunoglobulin G pooled from immunized human volunteers became available in a phase II clinical trial by the Orphan Drug Program of the FDA as a potential treatment for infant botulism (70). A similar pool of human antibodies could also be useful to alleviate side effects of botulinum toxin injections without leading to patient reaction to the antiserum.

Changes in muscle tissue following botulinum toxin type A injections. Changes in skeletal muscles after botulinum toxin type A injection have been studied in animal models (53, 56, 57, 155). Duchen (56, 57) found that muscle fibers became atrophied and sprouting of nerve fibers was induced after injection of toxin into the leg muscle of mice. Sprouting of motor nerves was observed after 6 to 7 days and progressed for several weeks in the red soleus muscle; it occurred later in the predominantly white gastrocnemius muscle. Nerve sprouting occurred as complex branched arrangements which were apparently unable to establish functional connections for several weeks. The muscle fibers atrophied for 6 weeks or more and then increased in diameter to within normal limits within a few weeks. Changes in the localization and intensity of cholinesterase staining reflected the morphological changes. This work was important because it provided a new approach to quantitative characterization of renervation of denervated muscle. Pestronk and Drachman (155) evaluated motor nerve sprouting quantitatively after presynaptic blockade with botulinum toxin by measuring acetylcholine receptors with ^{125}I-labeled α-bungarotoxin. Muscle disuse was maintained by repeated injections of tetrodotoxin. They showed that the amount of sprouting was correlated with the number of acetylcholine receptors and was greatest in the botulinum-poisoned muscles. Sprouting was inhibited by α-bungarotoxin, suggesting that the acetylcholine receptors had an important role in inducing sprouting and muscle renervation. These results suggest that the use of a combination of botulinum toxin and α-bungarotoxin could prolong muscle paralysis.

In an approach derived from that of Duchen (56, 57), Borodic et al. (23–26) have used the albino rabbit as an animal model to quantitatively determine toxin spread from the site of botulinum toxin injection. Acetylcholinesterase staining, muscle fiber size analysis, and ATPase staining were used to establish a denervation gradient. A gradient effect up to 30 mm from the site of injection of 2 to 3 U of botulinum toxin type A per kg was found with respect to morphological changes in muscle fiber size and histological staining. At distances greater than 30 mm, there was substantially decreased staining and much less muscle atrophy. Very similar results were found in a study with crude type B toxin (26). The denervation indicated by histochemical staining and fiber size analysis appeared transient and lasted for about 3 months for both type A and B toxins. By using muscle biopsies, innervation sites were also determined with humans (23–25). Borodic et al. (23) have also used electrical stimulation to determine motor points and optimal injection sites in botulinum toxin therapy.

Immunity to botulinum toxin. There is considerable concern about the possibility that patients will develop antibodies and become refractory to botulinum toxin treatment, particularly when relatively high levels of botulinum toxin are injected repeatedly over several years. The dose of toxin required to trigger antibody formation in humans is not known. The minute quantities of toxin ingested in foodborne botulism are not sufficient to evoke antibodies. Recurrent episodes of type B and type E botulism have been documented in the same individual, supporting the notion that repeated exposure to botulinum toxin may not impart long-term immunity (6, 186). Repeated sensitivity to tetanus toxin in humans has also been reported (34).

Toxoid is commonly injected into laboratory workers to

stimulate antibodies and protect against accidents. The minimum dose of toxoid to elicit immunity in humans varies greatly with the individual and the toxoid preparation (3, 80, 197), but is probably similar to the immunological response to tetanus toxoid (73). Repeated injections of botulinum pentavalent toxoid after 0, 2, and 12 weeks and yearly boosters gave final titers of 3.2 IU of anti-A antibodies, 0.4 IU of anti-B antibodies, and 2.5 IU of anti-E antibodies per ml. in a man (80). Antibodies were slow to develop, and a steep rise in the level of anti-A antibodies occurred in the fourth year of immunization. In an investigation of 77 patients subjected to the current U.S. schedule of toxoid injection at 0, 2, and 12 weeks, Siegel (197) reported that neutralizing antibodies to type A and B toxins were low or absent after the 12-week shot and significant titers were present only after yearly boosters. After the first booster, 74 (96%) had an anti-A antibody titer of 0.25 IU/ml or more, and only 44 (57%) of the subjects had an anti-B antibody titer of 0.25 IU/ml or more. (1 IU is defined as the amount of antibody neutralizing 10,000 MLD_{50}s.)

Antibody formation has been observed in a small number of patients injected with botulinum toxin (98, 191). To date, about 12 of more than 7,000 patients treated have developed antibodies to type A botulinum toxin. Six patients injected with 300 to 400 ng and one injected with repeated 100-ng doses within 30 days developed antibodies within 30 days (191). Antibodies have been demonstrated to reduce the beneficial effect of treatment (98). More work is needed to evaluate the incidence of antibody formation and other immunities in patients repeatedly treated with toxin over several years.

Properties and Uses of Serotypes of Botulinum Toxin Other than Type A

Seven known serotypes of botulinum toxin (A through G) have been isolated and characterized (213), and it is likely that types other than type A will be used clinically, particularly in patients who develop immunity to type A. Furthermore, evidence is accumulating to show that different types bind to different acceptors and may have subtle differences in their mode of action and that they could therefore complement type A in clinical applications. In the following sections, we review various basic science aspects of the botulinum toxins, especially as they pertain to potential clinical applications.

Botulism in humans. When botulinum toxin enters the circulation from contaminated food or infection, it can cause a severe paralytic disease. Types A, B, and E have most commonly been involved in human botulism (168, 213, 215), and type F has been the causative type in at least two outbreaks of food poisoning (78). Symptoms and severity of botulism differ depending on the serotype and amount of toxin ingested, suggesting possible differences in the mechanisms of intoxication (215). Clinical observations have indicated that type A food-borne botulism is often more severe and associated with higher mortality than botulism from other types (37, 52, 95). A rapid onset of neurologic signs indicates a more severe episode of the disease (38). Benign forms of botulism in which the course of the illness is milder and longer lasting have also been reported, particularly for type B (43, 100, 109, 209).

Botulism in humans generally manifests as a rapidly progressive symmetrical neuromuscular paralysis. Patients with botulism generally stay mentally alert during the poisoning unless anoxia sets in (108). Sudden respiratory or cardiac arrests and airway obstruction, leading to death, can occur (109). Cardiac effects of botulinum toxin in animals and in humans have been reported (114, 215).

Botulinum toxin most often initially affects eye muscles supplied by susceptible cranial nerves, and the first signs of botulism are often blurred and double vision (215). As the paralysis progresses and peripheral nerves are affected, signs such as dry mouth, difficulty in swallowing, weakness in head and neck movements, and difficulty in breathing become apparent. In type A and B botulism, loss of musculature control manifests as ptosis and drooping of eye muscles, hypoactive gag reflex, and weakness in upper and lower extremities (95). Atypical symptoms including asymmetric or late-onset of neurologic signs, paresthesia, nystagmus, ataxia, and sensory abnormalities are not uncommon (31, 95, 215). Ingested botulinum toxin can paralyze all muscles of the body. Symptoms of botulism sometimes last for months, and recovery requires renervation by new nerve terminal axons and end plates. Weakness and fatigability may persist for 1 to 2 years (215). Recovery in adults is generally complete (38), but there are reports of central nervous system involvement in infant botulism (100a).

Since botulism is rarely encountered, it can be difficult to diagnose rapidly. Electromyography is useful for detection of decreased amplitude of muscle action potential in weakened muscle (215), and since conduction along the nerve axons is not altered by botulinum toxin, the proximal motor nerve conduction rates and distal latencies are normal (42). Botulism is confirmed by the demonstration of botulinum toxin in the patient's serum or stool or in suspect food by mouse assay and neutralization with type-specific antitoxin (52). Botulinum toxin has been found more often in the serum of patients with type E or B botulism than with type A, possibly because of the greater affinity of type A toxin for tissue acceptors.

The actual dose of botulinum toxin to cause food-borne intoxication in humans is debatable and depends on the individual, the source and type of toxin, and the amount ingested. Accidental cases of human botulism from toxin-contaminated food showed symptoms of botulism and occasionally death from as little as 0.1 to 1 μg (100 to 1,000 ng or 3,000 to 30,000 MLD_{50}s) (134, 140, 183), but results were quite variable, probably because of individual variation in the amount absorbed and the stability of the toxin in the gut. More data on toxicity is available for lower animals and monkeys. The lethal dose of crystalline toxin type A in mice was 1.2 to 2.5 ng (0.03 to 0.07 U)/kg (76, 80) and was 0.5 to 0.6 ng/kg for guinea pigs and rabbits (76). Scott and Suzuki (193) determined that the intramuscular LD_{50} for juvenile monkeys (*Macaca fascicularis*) was ca. 39 U/kg (ca. 1.25 ng/kg) of body weight. Herrero et al. (91) reported a similar lethal dose of 40 U/kg by intravenous injection in *Macaca rhesus*. In Gill's table of lethal amounts of bacterial toxins, he reported botulinum toxin to be the most potent toxin known for primates, the lethal quantity of type A toxin being 0.5 to 0.7 ng/kg of body weight for monkeys and ca. 1 ng (30 U)/kg for humans (76). Larger quantities of types C_1, D, and E may be required to cause death in monkeys, whereas less type B is required (76). No data on intravenous toxicity are available for humans for botulinum toxins, but humans are probably as sensitive as guinea pigs and would be expected to be about as sensitive as monkeys.

Toxin production by the various serotypes of *C. botulinum*. Use of the various types of botulinum toxin in medicine will require a plentiful source of the toxins. The production of type A toxin under controlled conditions by the Hall strain,

as used for the preparation of toxin for human treatment, gives a uniform-crystalline toxin in high yields. The toxin complexes of the other types have also been obtained by culturing and purification and could be useful clinically. However, the strain, medium composition, and culture conditions affect the yields and structures of the botulinum toxins.

To obtain the greatest quantity and highest quality of toxin, it is essential to maintain strains of *C. botulinum* that consistently produce high levels of toxin. However, the bacterium has a frustrating tendency under laboratory conditions to gradually lose its ability to produce high levels of toxin. Lewis and Hill (118) reported that the Hall strain made decreasing quantities of toxin on successive subcultures. Huhtanen (96) also reported that strains of type A and B toxins frequently become nontoxigenic during culture. A more complete understanding of the physiological and genetic factors that control toxin production will be valuable for the development of other types.

The highest levels of toxin in group I *C. botulinum* (proteolytic strains of types A, B, and F) are generally produced in cell populations that undergo rapid autolysis and do not sporulate (21, 27), although Siegel and Metzger (198) obtained titers of 6.3 × 10^5 U with the Hall strain in a fermentor without appreciable cell lysis. Toxin formation is poor during sporulation, and spores contain only small quantities of toxin (ca. 1% of that found in cytoplasm) (58, 77). Takumi et al. (216) reported the isolation of nontoxigenic variants of *C. botulinum* type A that had enhanced sporulation. The strain used for production of type A, the Hall strain, sporulates very poorly. Therefore, encouraging vegetative growth and autolysis and discouraging spore formation may be important for obtaining good yields of toxin.

Toxin formation is controlled by nutrition in group I and II *C. botulinum* (119, 152). Arginine delayed autolysis, affected sporulation, and repressed toxin formation in group I *C. botulinum* (28, 154). Toxin formation was repressed about 10,000-fold in group I, including the Hall A and Okra B strains, when abundant arginine was available in the medium (152), probably owing to nitrogen repression of toxin gene expression. Protease was also decreased by arginine in group I *C. botulinum*. In group II *C. botulinum* (nonproteolytic strains of serotypes B, E, and F), tryptophan availability repressed toxin formation, probably also in response to nitrogen sufficiency (119). These results indicate that fermentation conditions and mutant strains could be developed for improved toxin production.

Significance of complexes on toxin quality. The strain and culturing conditions also affect the quality of toxin that is produced. Schantz and Spero (181) found that botulinum toxins of the different serotypes occur in spent cultures as large protein complexes. In the ultracentrifuge the sedimentation coefficients for the complexes were 19S for type A, 16S for type B, and 13S for types C, D, E, and F. Sugii and Sakaguchi (211) showed that high-molecular-weight toxin complexes occur naturally in foods. It is now known that each of the types of botulinum toxin produced in food or in culture are conjugated proteins ranging in molecular weight from 300,000 to 900,000, comprising a molecule made up of one or two neurotoxic units of about 150,000 M_r noncovalently conjugated to nontoxic proteins (168, 181, 213).

The formation of toxin complexes is very important for use of the toxins in medicine because the nontoxic proteins play an important role in maintaining the stability of the neurotoxic units. Isolated neurotoxic units were poorly toxic

to mice when administered orally (169, 170, 210). Peroral toxicity increased with incremental association of the neurotoxins with the protective proteins (150, 168–170, 210). The larger (19S and 16S) complexes of botulinum toxin types A and B were more toxic by the oral route and more resistant to acid and pepsin than were the smaller complexes. The isolated neurotoxins were rapidly inactivated by these conditions. Variations in the toxigenicities of different strains also probably depend on differences in the structures of the complexes. Ohishi (150) found that the oral toxicities differed considerably for the toxins of certain type A and B strains of *C. botulinum*. Of five B strains, Okra B produced the most potent toxin by oral challenge in mice. The 16S complex of the toxin was 700 times more potent than the 16S molecule from strain NH-2. A hybrid composed of the neurotoxin from NH-2 and the nontoxic components from Okra increased the oral toxicity close to that of the native Okra toxin, probably by protection of the neurotoxin in the gastric and intestinal tracts.

The size of the complex formed in types A, B, E, and F depends on the medium for bacterial growth. It has been known for years that some foods such as vegetables have high botulinogenic properties (134, 212). Sugii and Sakaguchi (212) showed that type A and B *C. botulinum* produced the stable 19S and 16S high-molecular-weight complexes in vegetables, whereas they produced the less stable 12S complex in tuna and pork. Nonconjugated neurotoxin was not found in any of the food substrates. They found that addition of iron or manganese to the growth medium resulted in a higher concentration of small toxin complexes (12S and 16S) in type A *C. botulinum*, suggesting an influence of metals on the size and stability of the complexes.

Biochemical and genetic properties of the neurotoxin component. The biochemistry of purified botulinum neurotoxins, particularly type A toxin, has been studied in considerable detail, and authoritative reviews are available (45, 81, 213). Neurotoxins have been purified for all serotypes except for type G; the type G toxin has been purified to a protein complex of high toxicity, but further purification resulted in substantial loss of toxicity (126, 146). Within a given type of toxin and strain of producing bacterium, there may be considerable heterogeneity in molecular structure and antigenicity, giving a mosaic structure (139). The neurotoxins all have high specific toxicities, from 10^7 to 10^8 MLD$_{50}$s/mg of protein (213).

All types of neurotoxins are synthesized as single-chain protein molecules of about 150,000 M_r with low toxicity. The protoxins are released from the bacterium during culture (48). Those of proteolytic (group I) *C. botulinum* strains are cleaved by extracellular proteases into two-chain molecules consisting of an H (heavy) subunit of about 100,000 M_r and a L (light) subunit of about 50,000 M_r (45, 47, 48, 171, 196). Toxin preparations from nonproteolytic cultures require exogenous protease treatment for protoxin activation. The H and L chains are covalently linked by at least one disulfide and noncovalent bonds (45) and possibly a metal component (10, 11). The H and L chains of the neurotoxins can be separated by chromatography after treatment with dithiothreitol and urea (171). The isolated chains are not toxic by themselves but can be recombined under carefully controlled conditions to obtain active toxin (110, 123, 214, 230). Recently a chimeric toxin which retained considerable activity was prepared between the L chain of tetanus toxin and the H chain of botulinum toxin type A (230). Chimeric toxins composed of defined fragments, e.g., the H chain from botulinum toxin and the L chain from ricin, could be

valuable in medicine, but much work needs to be done on their formation and clinical testing.

During proteolytic cleavage the neurotoxins undergo a molecular change in shape that increases toxicity (48). The nicking region was recently reported to contain multiple target sites susceptible to more than one protease (45, 47). DasGupta and Dekleva have proposed that two peptide bonds in a short region are cleaved at different rates during maturation of type A toxin and that 10 amino acids are excised (45, 47). The control of proteolysis to increase stability could be useful in the preparation of the toxins for medicine, as has been achieved with tetanus toxin, and to obtain defined fragments for construction of toxins with desired properties.

The presence of metals in neurotoxins may affect their stability. Bhattacharyya and Sugiyama (10, 11) reported that chelators for iron and manganese inactivated purified type A botulinum toxin and tetanus toxin. Analysis of purified botulinum neurotoxin for metal content by neutron activation indicated that one atom of iron was present for each toxin molecule. It was suggested that metals may be involved in linkage of the H and L chains of botulinum and tetanus neurotoxins (10, 11). Kindler and Mager (107) found that metal availability in the culture medium affected the formation of toxin. Culturing C. botulinum in a medium containing EDTA did not inhibit growth but completely suppressed toxin formation. The biological activity of botulinum toxin may depend on a transition metal component, possibly Fe. The presence of metals could be important in maintenance of activity and protection from oxidation during drying and for long-term stability.

Recent genetic advances have increased our understanding of the structure and expression of the botulinum toxins. The genes coding for botulinum neurotoxin types A, B, and E are present in one copy on the chromosome in representative strains (14, 219). Genomic libraries of C. botulinum type A chromosomal DNA (strain 62A or NCTC 2916) were prepared on plasmids and transformed into Escherichia coli. For safety reasons, separate subfragments that were 2 kb or less in size and did not encode the entire neurotoxin gene were cloned. Open reading frames which encoded a sequence corresponding to a polypeptide of 1,296 amino acid residues, 149,425 M_r (14) or 149,502 M_r (219), were identified. The nucleotide sequences were in agreement with the partial nucleotide sequence reported by Betley et al. (9). The promoter of the BoNT/A gene was not transcribed in E. coli; this may have been caused by the frequent presence of codons in the promoter region that are not normally present in E. coli. Codon usage in the botulinum toxin gene was similar to that previously found for the tetanus toxin gene (61, 65, 66). Overall, 90.3% of the degenerate codons ended in A or U. An exception to the codon bias occurred for Lys codons, in which the frequency of AAA and AAG was nearly the same (24 AAA and 20 AAG) (219) compared with 98 AAA and 9 AAG for the tetanus toxin gene. In C. botulinum, AUG and UAA were translational initiation and termination codons, respectively, and strong bias was found for Arg and Ser codons. Binz et al. (14) found that the A+T content in the 5'-noncoding region of the type A and type E toxin genes was 80.4 and 80.3%, respectively, higher than in the coding regions, where 73.6 and 72.1% A+T were found. Examination of the upstream region indicated that transcription started 118 to 127 nucleotides upstream from the translation initiation site (14). Regions of dyad symmetry were demonstrated in the 3' noncoding region that may be involved in regulation of transcription. Binz et al. (14) con-

cluded that botulinum neurotoxin type A was translated from a monocistronic RNA and that the mRNA did not also encode the hemagglutinin and other nontoxic proteins of the natural toxin complex. Thompson et al. (219) also concluded that a single open reading frame was translated, giving only the neurotoxin protein.

The sequence of the type A neurotoxin gene indicated that botulinum neurotoxin A does not possess a signal peptide in the terminal coding regions, supporting the notion that it is not a secreted protein. Cys residues are conserved at positions 1060 and 1280 of botulinum (and tetanus) toxins, and Cys-454 occurs at the same position in C. botulinum type A, B, and E toxins and in tetanus toxin (61). Cys-454 is the sole Cys residue in the N-terminal region of the H chain and is probably involved in disulfide bridging of the L and H chains. Cys-430 is also located at an identical position in botulinum and tetanus toxin L chains. Sequence analysis of botulinum type A toxin indicated that the H chain of type A neurotoxin had six histidine residues arranged in a motif which the authors suggested could be involved in the biological action of the toxin, possibly penetration through the nerve membrane. The deduced amino acid sequences of botulinum toxins had about 33% homology to tetanus toxin, and the H chains showed higher homologies than the L chains (14, 219). No homologies were detected to other proteins including ADP-ribosylating clostridial toxins.

The DNA sequences have also recently been obtained for other botulinum toxins including type D, C_1, and E neurotoxins and the C_3 ADP-ribosyltranferase (13, 71, 72, 87, 105, 106, 157). Highly homologous regions were detected among the various neurotoxin gene sequences and tetanus toxin gene. The C_3 gene was found to be unrelated to C_1 and D neurotoxin genes.

Structure and properties of nontoxic proteins of the toxin complex. Relatively little is known concerning the biochemistry and genetics of the nontoxic proteins associated with neurotoxin in toxin complexes. The type A complex contains at least two nontoxic proteins, one of which has hemagglutinating properties (115). Strains of C. botulinum that do not produce hemagglutinin have been isolated, and these form smaller complexes (12S and 16S) than are normally found (19S) (111, 135, 211). The in vitro addition of hemagglutinin to the 12S complex results in formation of a 19S complex with increased stability (111). Binding of the hemagglutinin was inhibited by a heat-stable, dialyzable substance that has not been isolated (211).

DasGupta (44) reported that the hemagglutinins of type A and B toxins were constructed through aggregation of two small units of about 15,000 and 20,000 M_r. Recently, Somers and DasGupta (204) studied nontoxic proteins from type A, B, C_1, and E toxin complexes. The proteins isolated from types A, B, and E had various degrees of hemagglutinating activity (Hn$^+$), while the protein from type E had no hemagglutinating activity (Hn$^-$). The type A Hn$^+$ and type B Hn$^+$ were serologically cross-reactive. Type A Hn$^+$, type B Hn$^+$, and type C Hn$^+$ were isolated as large aggregates (220,000 to 900,000 M_r), which were separated into multiple subunits of ≥17,000 M_r by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The type E Hn$^-$ of 116,000 M_r did not aggregate. The sequences of the 10 to 33 amino-terminal regions of the 17,000, 21,500, 35,000, and 57,000 M_r subunits of type A Hn$^+$ and type B Hn$^+$ were determined. Each of the subunits had a unique sequence, indicating that the subunits were not homomers of smaller units. The subunits types A and B had remarkably similar

sequence identity; i.e., the 21,500 M_r subunits were identical and the 57,000 M_r subunits had 80% identity.

An understanding of the genetics of the hemagglutinin component of the toxin complexes is also developing. Oguma et al. (148) showed several years ago that the capacity to produce hemagglutinin in *C. botulinum* type C was transferred by phages either separately or together with toxin. Physical linkage of a hemagglutinin gene and toxin gene was confirmed, and it was shown that the toxin and hemagglutinin genes were transcribed in opposite directions. Tsuzuki et al. (224) cloned the gene encoding the main component of hemagglutinin produced by *C. botulinum* type C. The complete nucleotide sequence of the gene indicated that it encoded a protein of 33,000 M_r. At 62 bp downstream from the termination codon of the cloned 33,000 M_r subunit of type C Hn$^+$ was an initiation codon followed by a coding sequence for at least 34 amino acids. Somers and DasGupta (204) found that the derived amino acid sequence of this open reading frame had 73 to 84% sequence identity with the 17,000 M_r subunits of type A Hn$^+$ and type B Hn$^+$ and significant similarity with the N terminus of type E Hn$^-$. This observation raises the interesting possibility that genes for some of the subunits have a similar genetic arrangement and a common ancestral origin. It is interesting that a sequence homology has been proposed between tetanus toxin and the hemagglutinin of influenza virus (138), indicating a possible viral origin of the neurotoxin.

New findings in the mechanisms of action of the different types of botulinum toxins. Botulinum neurotoxins A to G are antigenically distinct yet have a number of structural and mechanistic similarities. All of the neurotoxin types cause a chemical denervation at the myoneural junction by inhibiting acetylcholine release. However, there appear to be subtle differences in the mechanisms of action of the neurotoxins. Toxin types A and E, type B, and type F apparently bind to distinct high-affinity acceptor regions with similar affinities (K_D 10^{-9} to 10^{-10} nM) in synaptosomes and at murine neuromuscular junctions (20, 45, 62, 110, 128, 228, 233). Binding may occur in regions composed of sialosyl residues and protein (184) and may first involve low-affinity association of the H chain followed by high-affinity attachment. In addition to binding to different acceptor regions on the nerve surface, botulinum toxin types A and B have been reported to affect neurotransmitter release differently (74, 137). Electrophysiological studies have shown that type A affects asynchronous neurotransmitter release, whereas type B does not (137). Furthermore, an increase in the intracellular Ca$^+$ concentration by ionophore treatment reverses inhibition by type A but not type B in synaptosome preparations (5), and aminopyridine more readily reverses type A than type B inhibition at the myoneural junction (74).

Both the H and L chains of the neurotoxin may be required for poisoning in invertebrate systems (123, 158). In mammalian peripheral motor nerve terminals, the L chain alone is active after it is internalized (15, 49). The precise mechanism of blockade by the L chain is unknown, but it must affect a general and important component of the secretory machinery in various classes of neurons. Botulinum toxin blocks the release of several classes of neurotransmitters at central and peripheral neurons (15, 120). Recently, it has been proposed that the L chain may act at an intracellular membranous or cytoskeletal site to inhibit neurotransmitter release (5, 120). Because of the extraordinary toxicity of botulinum toxin, it is likely that it has enzymatic activity and acts catalytically or triggers a cascade of events that decrease neurotransmitter release. The intracellular

substrate of botulinum toxin remains an elusive grail that is being pursued by several laboratories.

An objective in treatment of hyperactive muscles is to prevent possible systemic reactions which could result from spread of toxin through the blood. Antibodies could be used therapeutically by application to the injection site to help limit the diffusion of toxin and alleviate side effects such as ptosis (190), or it may also be possible to add the nontoxic H chain after toxin injection to block toxin binding to neighboring nerves. The most desirable approach to avoid spread would be to confine the paralyzing action within the presynaptic nerve. An interesting recent development is the finding that stabilized mRNA (3' polyadenylated and 5' capped) corresponding to the nucleotide sequence of tetanus toxin gene (L chain) injected into *Aplysia californica* cholinergic neurons in a bath depressed neurotransmitter release in less than 1 h (136). Similar results were found for mRNA of the L chain of botulinum A toxin, but only when the H chain was also added to the bath. The L chains of tetanus and botulinum neurotoxins were demonstrated to be synthesized in the presynaptic neurons, and onset of toxin action was slower than that of neurotoxins injected directly.

The subtle differences in botulinum toxin mechanisms among the various serotypes suggest that combinations of botulinum toxins could be more effective in clinical practice than any one type alone. Further work is needed to produce, stabilize, and test the clinical effectiveness of different types. Preliminary work indicates that types B (26) and F (187) are useful in controlling certain spasmodic muscle disorders.

Clinical use of pure neurotoxin compared with toxin complexes. Most recent information concerning the structure and pharmacology of botulinum toxin has been obtained with purified neurotoxins, but it is unlikely that these will be used in a clinical setting. The toxin complexes are much more stable than neurotoxins and can be diluted and formulated with retention of toxicity. Pure neurotoxins can be kept for several weeks to months in solution in the cold but are inactivated on dilution, formulation, and drying. No clinical trials on primates have been performed with purified neurotoxins.

Sellin et al. (195) reported that injection of 1 to 20 U of crystalline type A botulinum toxin into the lower hindlimb of the rat produced a paralysis that lasted for several days. In contrast, injection of more than 1,200 U of type B neurotoxin was required to produce paralysis. The duration of paralysis was compared after injection of 20 U of type A or 5,000 U of type B toxin. Type A toxin caused paralysis for up to 7 days after injection, whereas type B toxin caused paralysis for only 3 days and twitching became evident at 5 to 7 days. It was also reported (104, 194) that pure type A neurotoxin was much more effective than type E or F neurotoxin in eliciting lasting paralysis in the lower hindlimb of rats.

Tetanus Toxin

Tetanus toxin, like botulinum toxin, is produced by an anaerobic sporeforming rod that has a similar morphology to *C. botulinum* (86). Unlike botulinum toxin, tetanus toxin can enter into the central nervous system by retrograde intraaxonal transport through motor nerves (17, 81, 132). It causes uncontrolled spasms of voluntary muscles by blocking the release of inhibitory transmitters including γ-aminobutyric acid and glycine (132). Tetanus toxin also has significant activity in decreasing acetylcholine release in cholinergic peripheral nerves when injected locally (54) and could possibly be used as an adjunct to or independently

from botulinum toxin for control of hyperactive muscles if the acquired immunity could be overridden. Tetanus toxin could also be used pharmacologically to transport substances to the central nervous system (17, 18). The biochemistry and pharmacological activities have been recently reviewed (17, 81). Here we consider aspects of the toxin that pertain to tetanus toxin production, stability, and potential use in medicine.

Crystalline structure. Tetanus toxin was originally purified by Pillemer et al. in the 1940s by precipitation methods. They obtained toxin crystals by carefully carrying out repeated precipitations in methanol and controlling the ionic concentration, pH, and toxin concentration (156). Tetanus toxin, like botulinum toxin, is a simple protein that does not contain lipid or carbohydrate (156, 165, 166). Unlike botulinum toxin, tetanus toxin does not occur complexed with protecting proteins and will not survive gastric passage or cause food poisoning in humans.

Although crystals of tetanus toxin were obtained in the 1940s by Pillemer et al. (156) from cold alcohol solutions, crystallization was not confirmed by others for several years. More recently, two-dimensional crystals of tetanus toxin have been isolated from ammonium sulfate solutions after incubation for several weeks at 4°C (40, 167). Robinson et al. (167) obtained two-dimensional arrays of native tetanus toxin formed at the interface between a solution of the toxin and a phospholipid monolayer containing ganglioside. Crystalline arrays were obtained only when all three components (toxin, phospholipid, and ganglioside) were present. The three-dimensional structure of tetanus toxin at 14-Å (1.4-nm) resolution appeared as an asymmetrical three-lobed structure that could interact with the phospholipid monolayer in two possible orientations (167). The analysis indicated that tetanus toxin is composed of differently shaped domains with different functions.

Biosynthesis and activation of tetanus toxin. Tetanus toxin is synthesized intracellularly as a single polypeptide of 150,00 M_r that is released from the cells on autolysis and is then modified by proteases present in the medium (81, 88, 89). The single-chain molecule is difficult to isolate (81), and proteolytic modifications of the toxin have caused considerable difficulties in the accurate characterization of the molecule. Single-chain toxin can be prepared from washed extracted bacterial cells (88, 162) and by inclusion of protease inhibitors and use of specific purification procedures (7, 165, 231). Purified preparations containing protease inhibitor can be stored for 4 to 6 weeks without proteolytic modifications and loss of toxicity (165).

Conversion of tetanus toxin to the nicked form increases toxicity (7, 81). *C. tetani* forms proteases that produce nicking in culture (231), but many other endoproteases will also activate the toxin (2). Three regions in the molecule are particularly susceptible to nicking (81, 129). Mild trypsin treatment of intracellular single-chain toxin yields two chains of about 95,000 and 50,000 M_r. The modified tetanus molecule is strongly held together by noncovalent bonds, and reduction of disulfide does not result in separation of the chains. Strong denaturants such as urea or SDS (81, 166) or purification techniques such as isoelectric focusing (2, 231) are required to dissociate the chains.

The H chain of tetanus toxin possesses a particularly susceptible region that can be cleaved with proteases such as trypsin or papain, yielding two fragments (B and C) (2, 89). The isolated H and L chains and fragments B and C are poorly toxic compared with intact tetanus toxin (2). Ahnert-Hilger et al. (2) reported that the nicking sites contributing to

toxicity are located within a region spanning no more than 17 amino acids, and the N and C termini are not altered during the modification. The separated chains were reconstituted to active toxin. By reconstitution experiments, the L chain was demonstrated to possess the paralyzing activity in isolated nerve-muscle preparations. The H chain is required for toxin entry into the nerve tissues and for axonal transport (2).

Large-scale production of tetanus toxin. Tetanus toxin is produced in deep culture by methods similar to those described for botulinum toxin. J. Howard Mueller, Pauline Miller, and associates at Harvard Medical School developed the methods currently used for production of tetanus toxoid. They experienced much frustration in obtaining consistent quality and the high titers of toxin required for toxoid demand (121, 142–144). They realized the importance of medium formulation in obtaining good-quality tetanus toxin, "If it were only possible to grow the tetanus organism on a medium containing only chemically defined substances of low molecular weight, it should become a relatively straightforward matter to study and control the factors involved in toxin production, and to obtain a uniform product free from any possible antigenic material other than the specific substance desired." (141). An extensive study was carried out to identify factors controlling tetanus toxin formation (143). On fractionation of components of the medium, the basis of good production was determined to be present in a pancreatic digest of casein. The key to good toxin production by *C. tetani* was later determined to be limitation of histidine (144). Abundant free histidine drastically decreased toxin production, while its limitation strongly increased titers (144). Since histidine is required for growth of *C. tetani*, it was necessary to find a method to limit the nutrient without stopping growth. Mueller and Miller found that providing histidine-containing peptides (e.g., glycyl-histidine) or histidine esters (e.g., acetyl-histidine) stimulated toxin production. Latham et al. (117) developed a protein-free medium which is currently used for tetanus toxin production. Mueller and Miller also isolated a high-producing strain (the Harvard or Massachusetts strain) (142) that is still widely used by many laboratories.

Tetanus toxin synthesis was found to be repressed by the addition of excess amino acids to the medium (223). Mellanby (131) reported that glutamate addition to the Mueller and Miller growth medium decreased toxin formation but shortened the time necessary for autolysis. The results indicate that nitrogen nutrition controls toxin regulation in *C. tetani*. It is interesting that excess nitrogen also represses botulinum toxin synthesis in *C. botulinum* (119, 152). Careful adjustment of the levels of iron salts in the medium is also necessary for good tetanus toxin production (67, 142). The mechanisms of nutritional regulation and its importance in the biology of *C. tetani* and *C. botulinum* have not been further studied to our knowledge.

Tetanus toxin, like botulinum toxin, is produced in highest quantities by nonsporulating cultures (85, 145). Highly toxigenic cultures autolyzed thoroughly and did not form endospores during culture. During culture, tetanus toxin was present within the cell and was not released until cultures lysed (141, 162). As with *C. botulinum*, it appears that toxin formation is associated with autolytic growth and inversely associated with sporulation (145, 153). It would be of interest to determine whether specific transcription factors, e.g., sigma factors, regulate transcription of the toxin gene and whether these are preferentially expressed or activated in autolytic growth compared with sporulation.

Genetics of tetanus toxin. Tetanus toxin production has

been recognized as an unstable property for many years (68, 142). Attempts were made early to correlate the toxigenicity with the presence of bacteriophage. Phages were induced in *C. tetani* by treatment with mitomycin C, but induction did not affect toxin production (159, 160). Nontoxigenic mutants were readily isolated at high frequency (0.8 to 3.2%) from the Harvard strain A47 by treatment with various mutagenic agents including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, UV light, and rifampin (85). Hara et al. (85) found that cured, nontoxigenic strains still carried phages and proposed that plasmids could be involved in toxigenesis. Laird et al. (112) showed that toxigenicity was associated with the presence of a single large plasmid in 21 strains of diverse origin. Nontoxigenic derivatives were isolated, and each strain lost its plasmid. Two naturally occurring nontoxigenic strains were examined, and one was free of plasmids while the other contained a single large plasmid (112). The strains derived from the Harvard strain all contain a plasmid of 49 kb, which had identical restriction nuclease digestion patterns (112). By construction of a pool of nucleotide probes corresponding to the *N*-terminal amino acid sequence of tetanus toxin, Finn et al. (69) located the tetanus toxin gene to plasmid-related sequences. Surprisingly, strains with deletions in the plasmid still hybridized to the probes, suggesting that toxin gene sequences were still present but were not expressed to active product.

Eisel et al. (61) used a pool of oligonucleotides (heptadecamers) made up of all possible DNA sequences for N-terminal amino acids 8 to 13 of fragment C of tetanus toxin and screened plasmid preparations from toxigenic and nontoxigenic variants of the Harvard strain. Overlapping sequences that spanned the entire toxin gene were obtained from eight clones. The DNA fragments encoding tetanus toxin specified an open reading frame of 1,315 amino acids of 150,700 M_r. The open reading frame begins with an initiation codon for methionine, but purified toxin, like botulinum toxin, possesses proline at its N terminus and contains serine at the N terminus of the H chain (61). The molecular weights of the H and L chains calculated from the amino acid sequence are 98,300 and 52,288, respectively. Partial sequences reported for peptide fragments from the L chain (166) are only partly consistent with those obtained by nucleotide sequencing. The discrepancy may be caused by the extensive proteolytic processing that tetanus toxin undergoes following cell autolysis.

Computer searches using the primary sequence of tetanus toxin have not revealed primary structural similarities with any proteins other than botulinum toxin. Analysis of the primary sequence has also provided evidence that tetanus did not evolve by duplication of sequences within the H and L chains, which was earlier suggested by the similarities in amino acid compositions of the H and L chains (217) and by immunological similarities probed with monoclonal antibodies (227).

The availability of cloned tetanus gene fragments has enabled the production of tetanus toxin fragments for potential use as vaccines. Makoff et al. (124) expressed tetanus toxin fragment C in *E. coli* as 3 to 4% of the total cell protein. However, the coding sequence for fragment C is A+T rich and contains several codons rarely used in *E. coli*. Production was improved by replacing the coding sequence by a sequence optimized for codon usage in *E. coli* (125). More efficient translation of the mRNA was the most important factor for the increased expression. When the modified coding sequence was combined with improved promoter strength, fragment C was expressed as 11 to 14% of the cell

protein. Halpern et al. (83) cloned the sequence encoding fragment C and showed that the fragment expressed in *E. coli* retained ganglioside- and neuronal cell-binding activity. Recombinant fragment C was purified in one step by affinity chromatography. Recombinant fragment C was also immunogenic in mice and elicited antibodies that protected against tetanus toxin challenge. The availability of recombinant fragment C should be useful for a variety of research applications and for production of toxoid.

Pharmacological and medical applications of tetanus toxin. Tetanus acts primarily in the central nervous system and causes hyperactivity of the motor system and a spastic paralysis. Under specific conditions, tetanus toxin also inhibits peripheral neuromuscular transmission, resulting in a flaccid paralysis (82, 130). Tetanus toxin resembles botulinum neurotoxin in its structure and mode of action (81). Botulinum and tetanus neurotoxins have significant homology at the amino-terminal regions of the L and H chains, suggesting that at least portions of the respective genes evolved from a common ancestral gene.

Similarities in structure of tetanus and botulinum toxins have also been demonstrated in serological studies. Antibodies to type C botulinum toxin cross-reacted with other botulinum toxin serotypes and also reacted with tetanus toxin (149, 225). Tsuzuki et al. (225) found that a monoclonal antibody raised against botulinum toxin type E cross-reacted with botulinum toxin types B, C_1, and D and with tetanus toxin. Halpern et al. (84) developed antibodies against defined regions of the tetanus toxin to identify regions shared by tetanus and botulinum toxins. Synthetic peptides that corresponded to different regions of tetanus toxin were prepared and coupled to bovine serum albumin, which were used to immunize mice. Eleven of 13 peptides elicited antibodies that reacted with tetanus toxin in an enzyme-linked immunosorbent assay. Of 10 anti-tetanus peptide antibodies that reacted well with tetanus toxin, 1 reacted with botulinum toxin types B, C_1, and E but did not recognize type A. This antibody was made with a peptide corresponding to the amino-terminal end of the tetanus L chain, suggesting that this region is important in intoxication and that its structure is conserved in the two toxins. The antigenic region may be shielded in the native toxin but exposed on denaturation. Halpern et al. (84) also tested human tetanus immune globulin and mouse anti-tetanus serum for cross-reactivity with botulinum toxin, but none was detected. These results suggested that native forms of tetanus and botulinum toxins have little common surface antigenicity. This conclusion was also reached by Tsuzuki et al. (225), who prepared 306 monoclonal antibodies against the L chain of botulinum toxin type E and found that only 1 reacted with the other botulinum toxin types and with tetanus toxin.

Tetanus toxin has the unique ability to enter into the central nervous system through motor neurons. Because of the ability to travel up motor nerves, tetanus toxin or nontoxic fragments could provide a unique neurotropic agent to transport substances to the central nervous system (16–19). A 45-kDa nontoxic fragment, B-II$_b$ (fragment C), that bound to toxin-binding sites on neuronal cell membranes and transported retrogradely from the axonal endings within the muscle to the motoneural perikaria was isolated (19). Bizzini et al. (18) constructed hybrid molecules consisting of the neurotropic fragment C and the I$_{bc}$ fragment derived from tetanus toxin connected through disulfide linkage. The I$_{bc}$ fragment was specifically carried to the central nervous system. Bizzini (17) also reported that fragment C

could compete with rabies virus for attachment to binding sites on neuronal cells and affected the rate of spread of rabies virus. Cloning and expression of high levels of fragment C should lead to further studies of targeted delivery to the central nervous system and possibly to control of virus infections.

Tetanus toxin also can act peripherally, causing a flaccid paralysis in the manner that characterizes botulinum toxin (54). H chains of both botulinum and tetanus toxins form channels in lipid bilayers (94). The H_2 fragment of the H chain of tetanus toxin was found to antagonize the action of botulinum toxin in phrenic nerve-hemidiaphragm preparations (201, 202). Tetanus toxin is about 2,000 times more toxic at central inhibitory nerves than at peripheral synapses (12, 54) and is about 1,000 times less toxic than botulinum toxin type A at the myoneural junction (82). Dreyer and Schmitt (55) proposed that tetanus toxin and botulinum toxin type A act at different sites in nerve inhibition of transmitter release. Botulinum toxins type B (74, 195), D (54), and F (104), but not A (74), appeared to act in a similar manner to tetanus toxin in affecting transmitter release from the myoneural junction. The combination of botulinum and tetanus toxins or the construction of chimeras could potentially be used to control neurological disorders.

MICROBIAL NEUROTOXINS THAT ALTER VOLTAGE-GATED SODIUM CHANNELS

Other microbial neurotoxins impair muscle activity in a way different from botulinum and tetanus toxins by their effect on the action potential at the sodium channel of a nerve axon. Saxitoxin and tetrodotoxin are two classical examples of microbial neurotoxins that block or close the passage of sodium ions through the channel. Toxins produced by other dinoflagellates also produce changes in the action potential at the sodium channel and are briefly described below.

Saxitoxin is a potent rapidly acting neurotoxin produced by the marine dinoflagellate *Gonyaulax catenella* (206) and some bacteria (102, 122). Like botulinum toxin, it was first observed as a food-borne toxin, causing food poisoning that occurred only at certain times from consumption of mussels, clams, and some other shellfish that are plankton feeders. Consumption of toxic shellfish results in symptoms described as numbness of the lips and fingertips within a few minutes followed by a progressive paralysis of the arms and legs along with the development of labored breathing and asphyxia. Death may occur within 2 to 24 h, depending upon the dose, from respiratory paralysis. After survival for 24 h the prognosis is good, and no lasting effects of the toxin have been observed. The oral dose that causes death from accidental consumption of toxic shellfish by humans is 1 to 4 mg (5,000 to 20,000 mouse units) depending upon the age and physical condition of the patient. A mouse unit (MU) is defined as the minimum amount needed to cause the death of an 18- to 22-g white mouse in 15 min, which is usually the maximum time in which death will occur (174, 205).

Saxitoxin was first purified and crystallized by Schantz et al. (172, 179), and its structure was determined by X-ray crystallography by Jon Clardy (175). Purified saxitoxin is a very hygroscopic water-soluble toxin and is described chemically as a tetrahydropurine base with pK_as at 8.5 and 11.5. It has a molecular weight of 299 as the free base. It has no UV absorption above 210 nm. As the dihydrochloride salt it is a white solid that is stable in acidic solution but loses activity above pH 7. The paralyzing action of saxitoxin or its

binding at the receptor of the sodium channel depends upon the presence of a hydrated ketone group in a particular position in the molecule. Reduction of this group to the alcohol results in the loss of over 99% of the binding and paralyzing activity.

The neurotoxic action of saxitoxin is due to its specific binding, even at extremely low concentrations (10^{-9} M), at the sodium channel of excitable membranes and preventing the passage of sodium ions through the sodium channel, thus blocking an impulse. The action or binding is concentration dependent, and binding is reversible. Controlled application of the toxin has been suggested as a possible local anesthetic. The effective dose in animals is relatively close to the lethal dose, as indicated by the steepness of the response curve, and pharmaceutical companies have not pursued its use in humans. However, when saxitoxin is mixed in small amounts with many classes of anesthetics, the effectiveness of the anesthetic action is greatly extended (1). The addition of 1 μg of saxitoxin to a 1% solution of a typical anesthetic (1 part in 10,000) such as procaine increased the time of effective action two- to threefold (1). The result is not an additive one (1). The addition of saxitoxin to procaine as well as to other anesthetic compounds will also reduce the dose required to obtain a desired effect. The reason for this unusual action of saxitoxin with anesthetic compounds has not been fully explained, but the molecule must play an important part in nervous function in the presence of other substances that act on the nervous system. Saxitoxin and tetrodotoxin have been important in the establishment and characterization of the sodium channel in myelinated and unmyelinated nerve membranes (90, 93, 102, 163, 164) and for the study of related diseases such as multiple sclerosis.

Another species of dinoflagellates, *Gonyaulax tamarensis* var. excavata, produces saxitoxin substituted with sulfate and sulfonic acid groups (63, 102). These substituted toxins have a lower specific toxicity or binding at the sodium channel than saxitoxin does, but they should not be overlooked for possible medical use.

Tetrodotoxin was originally found in the roe, ovaries, and liver of the puffer or globe fish (*Tetfaodontidae*) caught in the western Pacific ocean and was at first believed to be exclusively produced by this fish. More recently it has also been found in various other animals including the California newt, octopus, and frog (234) and in marine bacteria (51, 199, 220, 235). Dinoflagellates have been proposed as the original source of the toxin in puffer fish, which acquire it through the food chain (234).

The action of tetrodotoxin is like that of saxitoxin in blocking the sodium channel of excitable membranes of nerve and muscle tissue. In fact, it has been shown that both tetrodotoxin and saxitoxin block the inward current of sodium ions at equally low concentrations of 10^{-7} to 10^{-9} M and occupy the same receptor sites at the sodium channel (63, 103). The basic structure of tetrodotoxin is markedly different from that of saxitoxin and is chemically described as aminoperhydroquanizoline, with a molecular weight of 319. Although the two toxins are basically different in structure, they may be similarly classified as heterocyclic guanidines because of the guanidium group common to each toxin. Kao and Nishiyama (103) first proposed that the guanidinium moieties of each toxin might enter at the sodium channel like guanidine and that the bulk of the remaining part of the molecule prevented the passage of the sodium ion.

Although this hypothesis may be consistent with many aspects of the action of the toxins, it appears that the chemical makeup of the molecule as well as the guanidine

group is involved. The reduction of the hydrated ketone group to an alcohol in the saxitoxin molecule completely destroys its effectiveness as a blocker of the sodium channel, and changes in the structure of tetrodotoxin also affect its bonding (102). The purpose here is to point out in a general way the nature of the two toxins and how they might affect the action of other toxins used for treatment of hyperactive muscles. The fact that saxitoxin enhances the action of local anesthetics has raised some thoughts on the relation of one toxin to another on an excitable membrane. Reviews by Catterall (36), Kao et al. (102, 103), and Borison et al. (22) give detailed descriptions of the action of the microbial neurotoxins saxitoxin and tetrodotoxin (binding site 1 at the sodium-channel) and compare them with neurotoxins from other nonmicrobial sources that affect the sodium channel, such as veratridine, aconitine, batrachotoxin, grayanotoxin, and the low-molecular-weight basic polypeptide toxins isolated from scorpion venoms, fish-hunting cone snails, and sea anemone nematocysts (binding site 2 at the sodium channel). These reviews point out the various mechanisms by which toxins might affect the nervous system via action at the sodium channel. From the proposed action it seems reasonable to assume that there may be value in the use of combined toxins for control of nervous activity.

It is quite interesting that the action potential at the sodium channel is also affected by certain substances, such as guanidine and 3,4-diaminopurine, that reverse or bypass the blocking action of botulinum toxin. Although these substances are not particularly good antidotes for the toxin, their action indicates a relationship between the action potential at the sodium channel and the liberation of a neurotransmitter at the nerve ending. One might assume, therefore, that substances such as saxitoxin, tetrodotoxin, or others that alter the action potential at the sodium channel should warrant further investigation for possible medical application. Guanidine, an effective substitute for sodium in action potential generation in excitable membranes (93), is also reported to relieve symptoms of botulism (39), suggesting that there may be interactions of toxins at the myoneural junction, a field that warrants further study.

Besides the microbial neurotoxins described thus far, there are other, less well understood microbial neurotoxins that may be found valuable for nerve and muscle control mainly because of their action at the sodium channel. *Gymnodinium breve*, a marine dinoflagellate responsible for the Florida red tides and the tremendous fish kills in that area, produces several toxins, two of which are neurotoxins designated brevitoxins A and B, that have some action at the sodium channel (22). These two toxins are lipid-soluble polyethers with a molecular weight around 900. Brevetoxin A has an indirect action on the sodium channel in that it enhances channel activity in the presence of toxins that bind to receptor site 2 at the sodium channel, but not to receptor site 1.

Gambierdiscus toxicus, a tropical reef-dwelling dinoflagellate, produces several toxins, including one designated ciguatoxin, which opens voltage-dependent sodium channels in cell membranes (186). This toxin is a lipid-soluble polyether with a molecular weight of 1112 and is concentrated as it is passed up the food chain to large predatory reef fish consumed by humans. The disease in humans affects both gastrointestinal and neurological systems. The neurological symptoms usually begin within 24 h and may last a month or more, indicating nerve blockage or damage requiring regeneration of nervous tissue. Afflicted persons experience cir-

cumoral paresthesias, paresthesias or paralysis of the extremities, and muscle pain.

Natural blooms of the freshwater blue-green alga (cyanobacterium) *Aphanizomenon flos-aquae* which occur periodically in lakes of the northern United States and certain provinces of Canada have caused poisoning of farm animals from drinking the water. This organism produces several toxins including saxitoxin and neosaxitoxin. Another species of this group, *Anabena flos-aquae*, produces a substance that affects acetylcholine receptors in muscle membranes (35, 97). Some mycotoxins affect the nervous system in various ways. Slaframine, upon biological conversion to a quaternary amine, causes excessive salivation in farm animals and acts similarly to acetylcholine (30, 41). These organisms and other microorganisms produce other neural toxins, but little is known of their action and importance in pharmacology and physiology.

CONCLUSIONS

Butulinum toxin type A has been found useful for the treatment of many hyperactive muscle disorders by intramuscular injection, and the FDA has licensed the toxin for treatment of strabismus, blepharospasm, and hemifacial spasm. It is the first microbial toxin to be used for human treatment. Because it is injected into humans, purity is of prime importance and, therefore, during the production by culturing and purification, it must not be exposed to any substances that might be carried in trace amounts to the crystalline toxin and cause undue reactions in the patient. Injection of the toxin into muscle tissue has opened a new field of investigation into the action of the toxin on muscle and nerve tissue and has been beneficial to many humans who suffer from dystonias.

Types of botulinum toxin other than type A toxin and perhaps tetanus toxin may be useful for human treatment if patients develop immunity to type A toxin. Saxitoxin stimulates and prolongs the action of local anesthetics, suggesting the use of combined toxins for human treatment. Some microbiological toxins are described for possible use alone or combined with botulinum toxin for medical treatment.

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MICROBIAL
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Comparison of wild type with recombinant *Clostridium difficile* toxin A

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Abstract

Toxins A and B from *Clostridium difficile* are single-chain proteins of 308,000 and 270,000 Da, respectively. They possess transferase activity to monoglucosylate proteins of the Rho GTPase family whereby Rho, Rac, and Cdc42 are the canonical substrates. For application of these toxins as specific Rho GTPase inhibitors the highest possible purity is of crucial interest. We, therefore, expressed recombinant His-tagged toxin A using the *Bacillus megaterium* expression system. Specific antisera raised against the native toxin A from *C. difficile* and the recombinant toxin, respectively, showed identical sensitivity and specificity in Western blot and ELISA analyses towards both toxins. By comparison of both toxins in functional studies we showed that the recombinant toxin was about two times more cytotoxic than the native toxin, and the glucosyltransferase-activity of the recombinant toxin was even 10-fold increased. However, recombinant toxin A showed one essential difference to the classically purified one. The reported transferase-independent effect of toxin A to release *cytochrome c* from isolated mitochondria was not exhibited by the recombinant toxin A. This putative mitochondrial effect decreased with increased purity of toxin A, and was absent with recombinant toxin, strongly suggesting an clostridial contamination responsible. In summary, we tested the recombinant toxin A to be at least an adequate substitute for the native toxin, bearing the advantage of a rapid single-step purification and the absence of biological active contaminations.
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Keywords: Glucosyltransferase; *Cytochrome c*-release; Enzyme-deficient toxin A; Cytotoxicity; RhoA

1. Introduction

Large clostridial cytotoxins are valuable tools in cell biology due to their specific spectrum of protein substrates [1]. Toxin A (enterotoxin) and toxin B (cytotoxin) from *C. difficile* specifically inhibit Rho, Rac, and Cdc42, whereas lethal toxin from *C. sordellii* inhibits Ras, Rap, Ral, and Rac [2,3]. These toxins are acknowledged as tools for studying Rho GTPase-dependent signal transduction pathways. The toxins enter cells by receptor-mediated endocytosis to act cytosolically, and are thereby applied in whole cell experiments. We recently reported on the expression of the 308,000 Da-sized recombinant holotoxin A, which is biologically full active [4]. So far, basically separated domains of *C. difficile* toxins, i.e. N-terminal catalytic domain, transmembrane domain, and the receptor binding domain, were expressed in an *E. coli* system. Several reports show expression of recombinant fragments of toxin A [5–9] or B [10–14]. Although the toxin fragments reveal the correct domain-specific function of the native holotoxin, their use, however, is mostly restricted to application in in vitro-systems or as immunogen. Sporadic reports of a recombinant holotoxin A or B stressed the difficulties in expression of these large clostridial proteins in *E. coli*. Phelps and co-workers [15] were the first to clone the complete toxin A gene. Kink and Williams [10] cloned the gene for full-length toxins A and B, but stated in their discussion a protein expression in *E. coli* that is too weak to yield sufficient protein for an efficient immunization. Similarly, Tang-Feldman and co-workers [16] reported low cloning efficiency of the toxin B gene, but succeeded at least in cytotoxicity assays to prove the expression of a biologically active toxin. Pfeifer and co-worker [17] finally showed the value of a recombinant tagged holotoxin B. Having this tool at hand, the authors discovered a processing of toxin B during internalization where only the catalytic domain reaches the cytosol.

Among the well-known advantages of a recombinant protein one aspect has to be stressed: the recombinant

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holotoxin represents a starting point for modifications, e.g. exchange of specific domains, mutations, or the generation of a glucosyltransferase-deficient toxin A. In all cases, proceeding studies demand prior characterisation of the recombinant toxin, which has been done in the present study. Of great interest was the feature of toxin A to induce mitochondrial damage [18]. The effect of toxin A and B on mitochondria triggers apoptosis and, thus, gains importance in the understanding of the pathomechanisms of these toxins. He and co-workers [19] postulated a glucosyltransferase-independent effect, since the effect on mitochondria was observed before significant Rho-glucosylation was detected. They also performed a *cytochrome c* release-assay as a method to study a glucosyltransferase-independent effect of toxin A [20]. By performing various assays for cytotoxicity, glucosyltransferase activity, immunological features, and a glucosyltransferase-independent effect of toxin A we characterized the recombinant toxin A as a valuable substitution for the native toxin A from *C. difficile*.

2. Results

2.1. Recognition of toxins by polyclonal antibody

The recognition by specific antibody is an important feature of the characterization of recombinant proteins. Therefore, immunoblot, ELISA and inhibition of toxin A-induced cytotoxicity by neutralizing antiserum were performed. Antibodies against the native and the recombinant enzyme-deficient toxin A were raised as described under Section 4.2. In a Western blot study (Fig. 1A), the antiserum raised in rabbits against the UDP-dialdehyde-inactivated native toxin A (anti-TcdA) recognized all toxin A preparations, the native, the recombinant, and the recombinant enzyme deficient mutant (used as immunogen). The antibody was specific towards toxin A, and did not cross-react with toxin B from *C. difficile*. The cross-check showed a comparable specificity of the antiserum raised against the enzyme-deficient recombinant toxin A (anti-rTcdA$_{ed}$). The proteins of lower molecular weight (less than 310 kDa), which appeared in the lanes of the recombinant toxins, are toxin fragments, since they are also recognized by the antibody raised against the native toxin. Of more value in testimony of the correct conformation of the recombinant toxin is the ELISA, allowing recognition of epitopes of non-denaturated proteins (Fig. 1B). Both antisera (anti-rTcdA$_{ed}$: grey bars; anti-TcdA: black bars) showed comparable specificity and sensitivity, recognizing the recombinant as well as the native toxin A, but not toxin B. As expected, the antiserum against the recombinant toxin A$_{ed}$ contains antibodies that neutralize the effect of native and recombinant toxin A, but not of toxin B (Fig. 1C). Shown is the inhibition of cytotoxicity of the native toxin A when preincubated with anti-rTcdA$_{ed}$ serum. Cells treated with recombinant toxin A (60 pM) or with toxin B (60 pM)
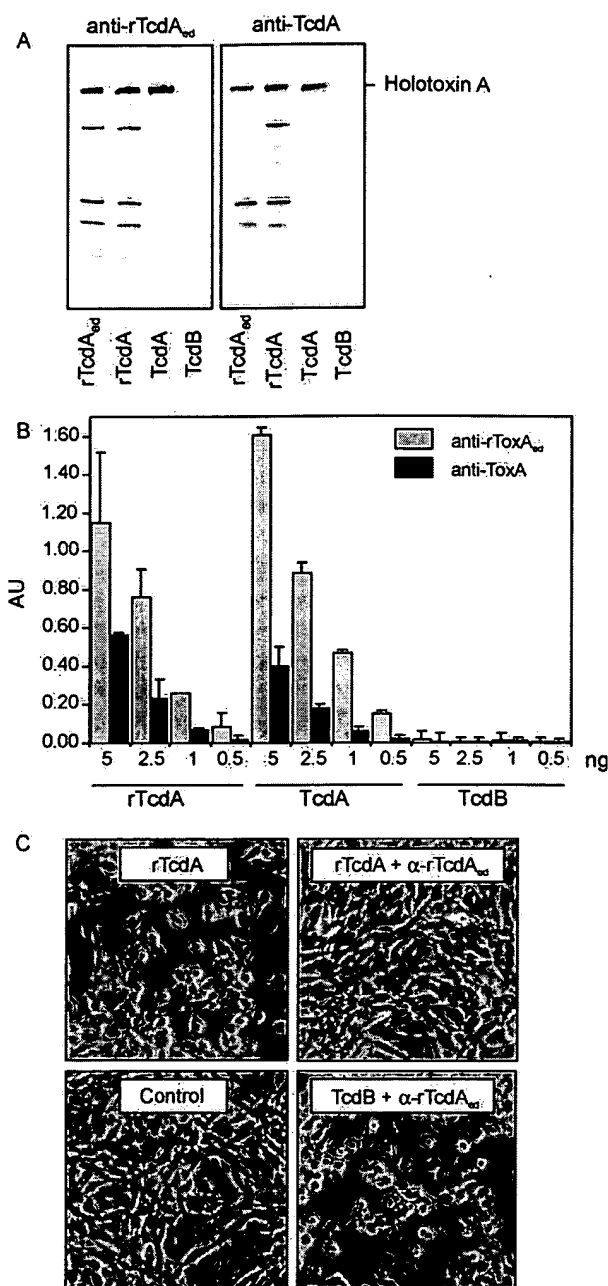

Fig. 1. Immunological characterization of toxin A preparations. (A) Western blot analysis of 10 ng each of enzyme-deficient recombinant toxin A (rTcdA$_{ed}$), recombinant toxin A (rTcdA), native toxin A (TcdA), and native toxin B (TcdB). The antisera raised against rTcdA$_{ed}$ and against TcdA both recognized all various toxin A preparations, but did not cross-react with toxin B. (B) Both antisera showed similar sensitivity in an ELISA in recognition of recombinant and native toxin A, but did not cross-react with toxin B. Shown are the arbituary units (mean values of quadriplicate samples ± SD). (C) Antiserum raised against the enzyme deficient recombinant toxin A prevented the cytotoxic effect of recombinant toxin A, but not of toxin B.

rounded up after 1.5 h. The anti-rTcdA$_{ed}$ serum completely prevented morphological changes of cells over an observation period of 10 h. The antiserum did also block the effect of the native toxin A (data not shown), but failed to inhibit the toxin B effect.

2.2. Cytotoxicity assay

The second set of experiments showed the comparison of the native and recombinant toxin A in a cytotoxicity assay. Native toxin A was prepared from the culture supernatant of *C. difficile* and the recombinant one was prepared from a *B. megaterium* expression system. To avoid artefacts caused by freezing/thawing, only freshly prepared toxins were used for this comparison. Fig. 2A shows the SDS-PAGE of both toxins. NIH 3T3 fibroblasts were treated with both concentrations of 30 and 15 pM of either native or recombinant toxin A. The morphological changes of cells were recorded hourly over a period of 5 h, and round cells were counted from three separate experiments. The kinetic of morphological changes induced by native and recombinant toxin A is depicted in Fig. 2B. After 5 h of treatment with 30 pM of recombinant toxin A cells were almost completely round, whereas the native toxin induced rounding of less than 50% of cells. A two-fold higher cytotoxicity of the recombinant toxin compared with native toxin A was also seen when 15 pM concentrations were applied.
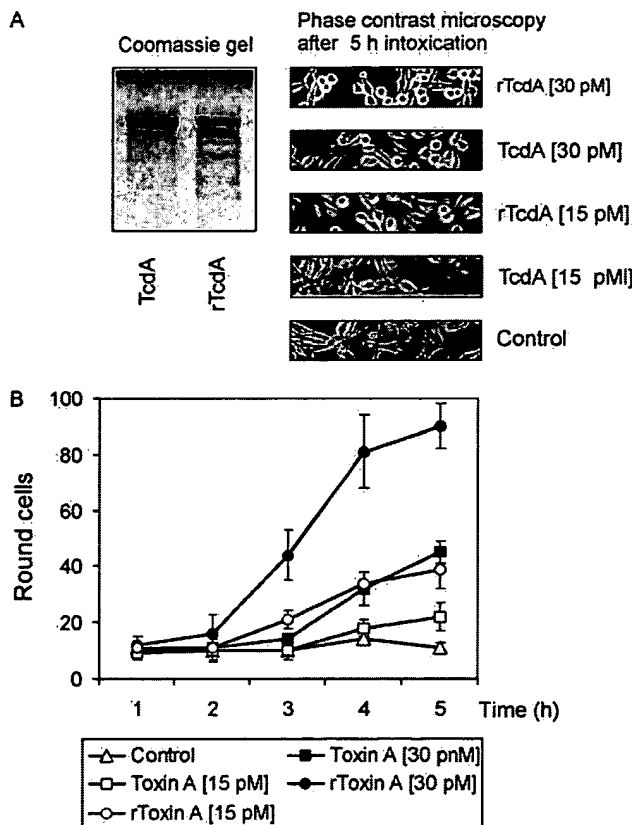
Fig. 2. Cytotoxicity assay. (A) Concentration of native toxin A and recombinant toxin A were adjusted according to SDS-PAGE and applied onto NIH 3T3 fibroblasts at concentrations as indicated. Rounded cells after 5 h incubation were recorded by phase contrast microscopy. (B) Graphical presentation of calculated cells that were completely rounded at indicated time points (mean values ± SD, n=3).
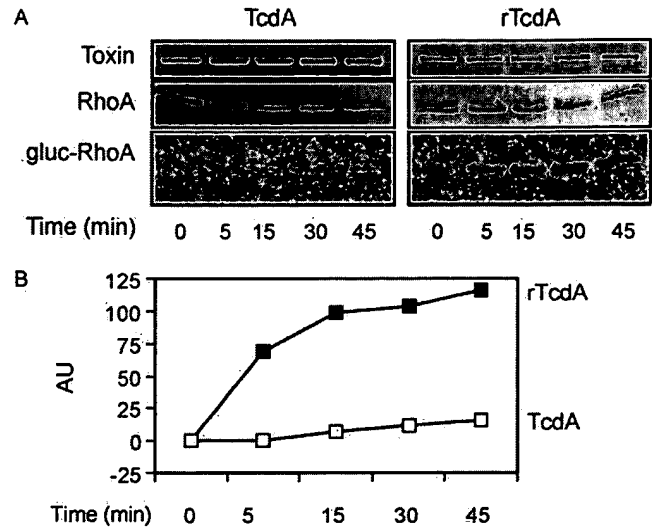
Fig. 3. Glucosyltransferase assay. (A) Glucosyltransferase assay was performed as described under Section 4.2 and SDS-PAGE of all samples was performed. The Coomassie gel shows the amount of toxin A of all samples (upper panel), RhoA (middle panel), and the phosphorimaging data of the [^{14}C]glucosylated RhoA (bottom panel). (B) Graphical presentation of the phosphorimaging data.

2.3. Comparison of glucosyltransferase-activity

A difference in potency of both toxins was more obvious in respect to glucosyltransferase activity. Fig. 3A shows the Coomassie-stained gel of all samples from [^{14}C]glucosylation with native toxin A (20 nM) and recombinant toxin A (10 nM). The upper lane gives the amount of toxin A in each sample, the middle lane the amount of RhoA, and the lower lane are the phosphorimager data of [^{14}C]glucosylated RhoA. The native toxin A revealed weak glucosylation of RhoA over a time course of 45 min. The recombinant toxin A catalyzed strong [^{14}C]glucosylation of RhoA even after 5 min, and almost maximum glucosylation after 15 min. The graphical presentation of the densitometrical evaluation is given in Fig. 2B. Taken into account that only half the amount of recombinant toxin A compared to native toxin A was applied, it can be assumed that recombinant toxin A exhibits a more than 10-fold higher glucosyltransferase activity than the native one.

2.4. Mitochondrial cytochrome c-release assay

In addition to cytotoxic and enzymatic activity as well as immunological characterization, a postulated enzyme-independent effect was used to further characterize the recombinant toxin A, namely the property to induce *cytochrome c* release from mitochondria. Mitochondria prepared from CHO cells were treated with 2 nM of different toxin A-preparations: a crude preparation (the elution fraction from the ion exchange column), an affinity-purified toxin A (from thyreoglobulin column), and the recombinant one. Whereas, the crude preparation
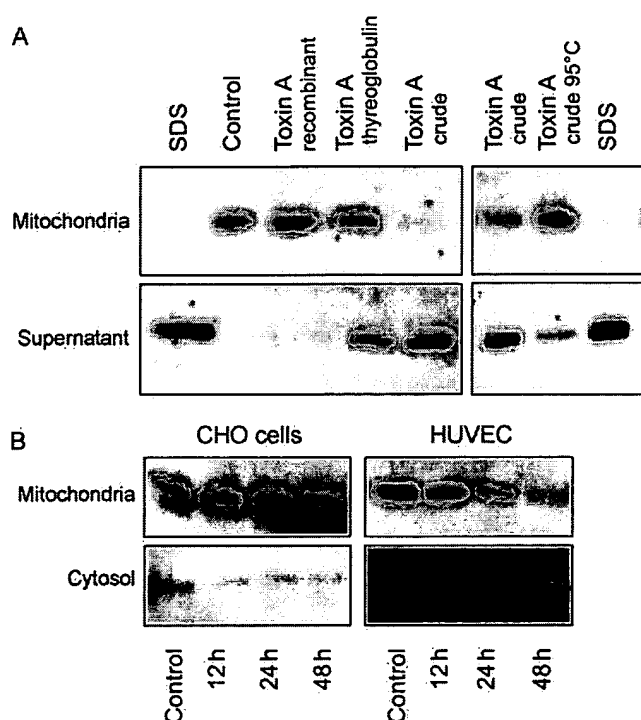
A

B

Fig. 4. Effect of toxin A preparations on isolated and cellular mitochondria. (A) Release of *cytochrome c* from isolated CHO mitochondria after treatment with 2 nM of recombinant toxin A, thyreoglobulin purified toxin A, and crude toxin A (upper panel). SDS was used as positive control for mitochondrial damage. The increase of *cytochrome c*-content of the supernatant is shown in the lower panel. Heating of crude toxin A for 10 min at 95 °C abolished the effect on mitochondria. (B) Effect of 2 nM crude toxin A on mitochondria in whole cell experiments with CHO cells and HUVEC. Mitochondria in CHO cells remained unaffected over a period of 48 h, whereas, mitochondria in HUVEC showed a *cytochrome c* release after 24–48 h. The *cytochrome c* was released into the cytosol of cells.

caused full release of *cytochrome c* within 90 min, affinity purified toxin A was less effective and recombinant one was without effect on mitochondria (Fig. 4A). Thus, the crude preparation seems to contain at least one factor, whose concentration was reduced by further purification steps, but was absent from the *B. megaterium* preparation. This factor was heat sensitive (10 min, 95 °C) suggesting a proteinaceous nature. Sodium dodecylsulfate (SDS, 0.01%) was used as positive control for complete release of *cytochrome c* from mitochondria into supernatant.

Cytochrome c was not released from mitochondria when whole CHO cells were treated with 2 nM crude toxin preparation over a period of 48 h (Fig. 4B, upper lane). HUVEC's (primary cells) were more sensitive to toxin treatment, and a *cytochrome c* release was observed after 24–48 h of treatment (lower lane). The *cytochrome c* which was released from mitochondria was detected in the cytosol. Thus, crude toxin fraction had no rapid effect on mitochondria in intact cells, possibly because of loss of contaminant during cell entry.

3. Discussion and conclusion

The present characterization of recombinant toxin A with respect to cytotoxicity, enzyme activity, and immunological characterization reveals equivalency to wild type toxin A. Two features, i.e. higher activity of the recombinant toxin A with respect to glucosyltransferase activity and cytotoxicity, and a different effect on isolated mitochondria, may be discussed in more detail to appraise both toxin A. In this study the recombinant toxin A was more potent in cytotoxicity as well as glucosyltransferase compared to native toxin A. As shown by Western blot analysis against His-tag, all major toxin fragments seen in SDS-PAGE are N-terminally truncated [4]. Since, N-terminal truncation of the first 15 amino acids results in a glucosyltransferase-deficient toxin (unpublished data), the fragments seen in Figs. 1A, 2A, and 3A are biologically inactive. Nevertheless, the fragments of the recombinant toxin were taken into account for adjustment of toxin amounts. Although the activity of toxin A varied between different preparations, we always observed higher toxicities of the recombinant toxin, which ranged from two-fold as reported previously [4] to 10-fold as found in the present study. The obvious reason for the higher activity of the recombinant toxin is the time-saving procedure of toxin preparation. The short-time preparation has the advantage that denaturation is less likely after 4 h than after 3 days. Both toxin preparations are summarized in a diagram, which clearly shows steps and duration of each preparation (Fig. 5). The advantage of preparation of the native toxin is the simultaneous preparation of toxin B, and a two-fold higher yield of toxin A per litre of *C. difficile* culture compared to *B. megaterium* culture.

It cannot be excluded that additional clostridial factors, which might restrict the activity of the native toxin A are co-purified from clostridium cultures. However, a supposed co-purification of associated proteins from clostridial culture supernatants gains more importance in the context of the mitochondrial *cytochrome c* release. As shown in Fig. 4 the crude toxin preparation induced a release of *cytochrome c*, which was less effective, when further purification of the toxin was performed. Toxin B, which was prepared in parallel to crude toxin A did also cause mitochondrial damage (data not shown). In general, thyreoglobulin affinity-purified toxin A did cause only weak leakage of *cytochrome c*, proving a loss of contaminants by further purification. This fact and the fact that recombinant toxin A has no effect on isolated mitochondria provides evidence that the glucosyltransferase-independent effect is mistakenly attributed to toxin A. It is not likely that the recombinant toxin A is less potent in mitochondrial *cytochrome c*-release, since, it exhibited higher activities in cytotoxicity and glucosyltransferase activity. We suppose that crude toxin A fractions are contaminated with a so far unidentified factor. This factor which perforates mitochondrial membranes is heat
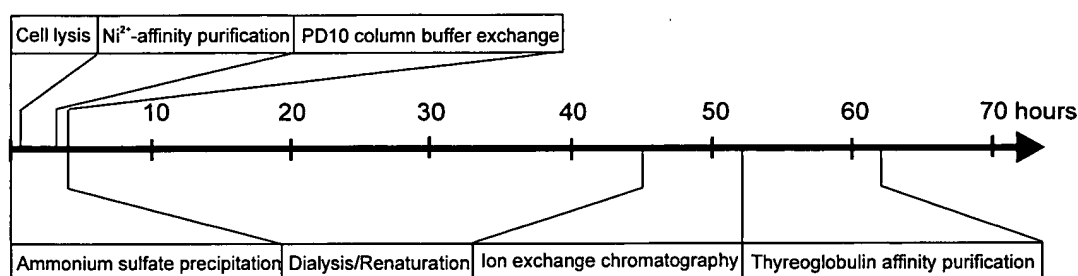
Fig. 5. Scheme of purification steps and duration of recombinant and native toxin A. Preparation of recombinant toxin A is performed within 4 h with a yield of 0.75 mg/l culture. Preparation of native toxin A lasts 3 days, resulting in toxins A and B in parallel, each of 2 mg/l culture.

sensitive as shown in Fig. 4, and thus, is of proteinaceous nature. A candidate for this contaminant is a holin-like protein. The holin-like protein has high homology to phage-coded holins, which lyse bacterial membranes. The production of a holin-like protein during toxin production of *C. difficile* strain 10463 [21] and its role in release of toxins were recently described [22]. Interestingly, this protein of 19 kDa is encoded in the tcdE gene, which is localized together with tcdA (toxin A gene) and tcdB (toxin B gene) in the pathogenicity locus. Tan and co-workers reported the recombinant expression by *E. coli* which resulted in cell arrest and lysis of bacteria, before a noteworthy production of protein was achieved. This implicates an even faint contamination of a holin-like protein in the toxin preparation of *C. difficile* as reason for a mitochondrial damage in a *cytochrome c*-release assay.

The two benefits mentioned above—time saving purification and a toxin preparation free of clostridial contaminants—are reasons for the replacement of the classically purified toxin A in further studies.

4. Materials and methods

4.1. Materials

Cell culture media were obtained from Biochrome, Germany, except Endothelial SMF medium, which was purchased from Gibco, Germany. HUVECs were from PromoCell, Germany. Thyreoglobulin, UDP-dialdehyde, Freud's adjuvant, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) were from Sigma. HiTrap Chelating HP and PD10 columns were obtained from AP Biotech, Germany. Quickchange XL site-directed mutagenesis kit was from Stratagene, Germany. *B. megaterium* protoplasts were from MobiTec, Germany. Monoclonal anti-*cytochrome c* (clone 7H8.2C12) was purchased from BD Pharmingen, Germany and peroxidase conjugated anti-rabbit was from Rockland, PA, USA. UDP-[^{14}C]glucose (300 mCi/mmol) was from Biotrend, Germany. All other chemicals were of the highest purity available.

4.2. Methods

4.2.1. Cell culture

NIH 3T3 fibroblasts, used for cytotoxicity assay, were cultured in Dulbeccos modified medium. HUVECs were cultured in Endothelial SMF medium, supplemented with 20 ng/ml bFGF, 10 ng/ml EGF, and 1 µg/ml heparin. CHO cells were kept in DMEM/HAM's F-12 mix supplemented with 1 mM Na-pyruvate. All media were additionally supplemented with 10% fetal calf serum, 100 U/ml streptomycin, and 100 µg/ml penicillin. All cells were subcultured twice a week.

4.2.2. Toxin preparation

The methods of different toxin preparations were described in detail by Just et al. (toxin A and B), Krivan et al. (thyreoglobulin affinity-purification), and Burger et al. (recombinant toxin A) [4,23,24]. In brief, native toxins A and B were purified from *C. difficile* culture supernatants by ammonium sulfate precipitation followed by ion exchange chromatography. Subsequent thyreoglobulin affinity-purification was performed to further purify toxin A. Recombinant toxin A was expressed as His-tagged protein using the *B. megaterium* expression system. The His-tagged toxin A was purified from the soluble fraction by a single step affinity chromatography using HiTrap Chelating HP column loaded with Ni^{2+}. The elution buffer of each toxin preparation was removed by a PD10 column, and toxins were stored in 50 mM NaCl, 20 mM Tris–HCl pH 7.4, 100 µM MnCl$_2$, and 20% glycerol at −80 °C.

4.2.3. Cytotoxicity assay

NIH 3T3 fibroblasts were seeded on a 24-well plate at a density of approximiately 10^5 cells per well. The cells were kept over night in culture medium and intoxicated the following morning. Non-frozen, freshly prepared toxins were used for comparison of cytotoxicity of original and recombinant toxin A. The toxins were added directly to the culture medium in concentrations as indicated in triplicate samples. Photographs were taken every hour to follow the morphological changes. Round cells were counted and comparative time courses for 5 h of intoxication were illustrated in a diagram.

4.2.4. Glucosyltransferase assay

The glucosyltransferase assay was performed as described by Genth and co-workers [25]. Recombinant RhoA (2 µg) in 100 µl glucosylation buffer (100 mM KCl, 50 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 1 mM $MnCl_2$, 10 µM UDP-[^{14}C]glucose [300 mCi/mmol specific activity]) was incubated for 45 min at 37 °C. The glucosylation reaction was started by addition of either native toxin A (3 µg) or recombinant toxin A (1.5 µg). Samples were taken at indicated time points and the reaction was stopped by addition of Laemmli buffer followed by 3 min boiling at 95 °C. SDS-PAGE and subsequent filmless autoradiography (Cyclon, Canberra Packard) was performed for documentation of [^{14}C]glucosylated RhoA. OptiQuant software (Canberra Packard) was used for quantification of detected signals.

4.2.5. Isolation of mitochondria and cytochrome c release-assay

Mitochondria of CHO cells were isolated by differential centrifugation as described by Perfettini and co-workers [26]. In brief, CHO cells were harvested in sucrose buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES (pH 7.5), 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin), and solubilized by repeated suction through a syringe (0.8×44 mm). The homogenate was centrifuged at $3.000 \times g$ for 10 min at 4 °C. The supernatant was used for preparation of the mitochondria enriched fraction by centrifugation at $10.000 \times g$ for 10 min at 4 °C. The pellet containing the mitochondria was resuspended in sucrose buffer to perform cytochrome c release-assay. To this end, the mitochondria solution was adjusted to a protein concentration of 15 mg/ml, and aliquots containing 30 µg protein were treated with each toxin (15 nM) or as indicated. After 90 min incubation at 30 °C mitochondria were pelleted by centrifugation at $10.000 \times g$ for 10 min at 4 °C. Samples from the supernatant and the mitochondria containing pellet were subjected to 15% SDS-PAGE and subsequent immunoblot analysis was performed using specific cytochrome c antibody. Preparation of mitochondria from whole cell assay was identical. The $> 10.000 \times g$ fraction of the cell lysate was used as cytosolic fraction to detect released cytochrome c.

4.2.6. Preparation of toxin A-antibodies

Rabbit polyclonal sera were used for the immunodetection of native and recombinant toxin A. Two approaches avoiding the lethal effect of toxin A as immunogen when applied i.v. led to the raise of specific antibodies: the use of original toxin A, which was chemically inactivated by UDP-dialdehyde [27], and the use of recombinant toxin A, where the DXD motif was mutated to generate a glucosyltransferase deficient holotoxin. Generation of an enzyme-deficient glucosyltransferase was described by Busch and co-workers [28] for the lethal toxin from C. sordellii. Mutation of the DXD motif from toxin A was

performed by Quickchange XL site-directed mutagenesis kit using 5′-GGCGGAGTATATTTAAATGTTAATAT-GCTTCCAGGTATTCACTCC-3′ (sense) and 5′-GGA-GTGAATACCTGGAAGCATATTAACATTTAAATAT-ACTCCGCC-3′ (antisense) primer for amplification. The whole expression vector containing the CDA gene (pWH1520 CDA$_{His}$, ~16.000 bp) was used as template for PCR. Immunization of rabbits were performed after standard protocol using the native as well as the recombinant toxin A, which both were glucosyltransferase deficient, in Freud's adjuvant. Application of the immunogen was sub-cutaneous for three times every third week.

4.2.7. Western blot and enzyme-linked immunosorbent assay (ELISA)

Western blot was performed using antisera in a 1:1000 dilution in TBS-Tween (0.2%). Peroxidase conjugated anti-rabbit was used as secondary antibody in a 1:2000 dilution in TBS-Tween (0.2%). The ELISA was performed as follows: a microtiter plate was incubated with indicated amounts of each toxin in PBS. The plate was agitated for 60 min at room temperature. After three times washing with TBS-T, 50 µl per well of indicated antiserum (1:2000, in TBS-T) were incubated for 60 min at room temperature. The plate was washed three times, and incubated for additional 60 min with 50 µl peroxidase-conjugated secondary antibody in a 1:1000 dilution in TBS-T. Again, the plate was washed three times with TBS-T. Detection of bound antibody was done by incubation of 50 µl per well 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as substrate for 30 min at room temperature. The absorption was read on a microplate reader at 405 against 620 nm.

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